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Mitochondrial Abnormalities in Remote Tissues of Patients with Amyotrophic Lateral Sclerosis

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative condition which is almost universally fatal. Some sufferers have an identifiable genetic mutation (<2%), but the majority of cases are sporadic (SALS). There is a body of evidence suggesting involvement of oxidative stress and mitochondrial abnormalities in the pathogenesis of ALS.

Mitochondrial function was assessed in remote tissues (muscle, cultured myoblasts, cultured fibroblasts) from patients with ALS and controls. Muscle tissue was examined for histological features consistent with a mitochondrial disorder. Mitochondrial respiratory chain function was measured in mitochondrial homogenates. Mitochondrial protein expression was determined using immunofluorescent antibodies to a mitochondrial DNA (mtDNA)-encoded respiratory chain enzyme subunit with image analysis. MtDNA quantity and quality was assessed using Southern blot and long-range PCR. Analysis of inheritance patterns was performed using a database of familial ALS cases. No mitochondrial abnormalities were identified using these techniques. There was no evidence of anticipation or a sex effect on inheritance.

In order to look for a difference in susceptibility to oxidative stress, cell cultures were examined for markers of oxidative damage and apoptosis, following growth with a free radical generating agent (paraquat). These studies demonstrated an increased susceptibility to oxidative damage in patient myoblasts and fibroblasts. This effect was not seen in cybrid studies combining mtDNA from patient platelets with experimental cells devoid of mtDNA (ρ^0 cells), where no difference in oxidative damage was seen, confirming the absence of a systemic mtDNA abnormality.

These results suggest that patients with SALS have an increased systemic sensitivity to oxidative stress which becomes pathological in tissues, such as motor neurons, which are considered to be vulnerable to such stress due to their metabolic activity and structure. This altered sensitivity is due to a factor other than a mitochondrial DNA abnormality, such as a nuclear DNA mutation or an unidentified environmental factor.

Abbreviations

ALS – Amyotrophic lateral sclerosis

APEX - Apurinic/apyrimidinic endonuclease

ATP – Adenosine triphosphate

bp – Base pair

BrdU – 5-Bromo-2'-deoxy-uridine

BSA – Bovine serum albumin

CI – Confidence interval

CNS – Central nervous system

CSF – Cerebro-spinal fluid

COX – Cytochrome oxidase

COX I – Cytochrome oxidase subunit I

DAPI – 4',6-diamidine-2-phenylindoldihydrochloride

dATP - 2'-deoxyadenosine 5'-triphosphate

dCTP - 2'-deoxycytidine 5'-triphosphate

dGTP - 2'-deoxyguanosine 5'-triphosphate

ddH_2O – Double-distilled water

DMEM – Dulbecco's modified Eagle medium

DMSO - Dimethyl sulphoxide

dNTP - deoxyribonucleoside triphosphate

dTTP - 2'-deoxythymidine 5'-triphosphate

DTNB - 5,5'-dithiobis(2-nitrobenzoic acid)

EDTA – ethylenediaminetetra-acetic acid

ELISA - Enzyme linked immunosorbent assay

FALS – Familial amyotrophic lateral sclerosis

g – Gravitational force

g – Grams

G_1 – Generation 1

G_2 – Generation 2

HB – Homogenisation buffer

H+E Staining – Haematoxylin and eosin staining

HSP – Hereditary spastic paraplegia

K – Pseudo first-order rate constant

kb – Kilobases

lrPCR – Long range polymerase chain reaction

min – Minutes

MND – Motor Neuron Disease

MnSOD – Manganese SOD

MPTP - 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine

MRC – Medical Research Council

mtDNA – Mitochondrial DNA

MTT - [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]

NADH - Nicotinamide adenine dinucleotide

NADP - Nicotinamide adenine dinucleotide phosphate

NMDA - N-methyl-D-aspartate

nDNA – Nuclear DNA

OXPHOS – Oxidative phosphorylation

PBS – Phosphate-buffered saline

PCD – Programmed cell death

PCR – Polymerase chain reaction

PCD – Programmed cell death

PMA – Peroneal muscular atrophy

PNS - Post nuclear supernatant

ROS – Reactive oxygen species

rpm – Revolutions per minute

S - Seconds

SALS – Sporadic amyotrophic lateral sclerosis

SD – Standard deviation

SDS - Sodium dodecyl sulfate

SMA – Spinal muscular atrophy

SOD1 – Superoxide dismutase 1

TAE – Tris, acetic acid, EDTA

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1 Introduction

1.1 Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) was first described by Charcot in 1869, with a clinical description of 2 cases and pathological description of 5 post mortem examinations (1). ALS is also known as Motor Neuron Disease (MND) and, in America, as Lou Gehrig's disease, after a well known baseball player who contracted the condition. ALS is a neurodegenerative disease of adult onset. In the UK, ALS has an incidence of around 2:100 000 annually and a prevalence of 6:100 000 (2). ALS occurs from the age of 20 with increasing prevalence with increasing age. The clinical pattern of ALS is that of a progressive disease affecting the lower (distal to the spinal cord) and upper (brain and spinal cord) motor neurons with sparing of the sensory system (figure 1.1). Weakness of the muscles occurs, along with spasticity. Immobiltity, loss of speech and swallowing may occur. Any muscle groups may be affected, and death most commonly comes about from respiratory involvement within 5 years of the onset of the disease (3). There has been, as yet, no specific environmental cause for ALS identified and the underlying pathogenesis of the sporadic disease remains to be identified.

Although there is a large degree of heterogeneity in the presentation of the motor neuron diseases, the presence of upper motor neuron signs and symptoms usually distinguishes ALS from the purely lower motor nerve disorders, such as spinal muscular atrophy. The diagnosis is usually made by finding the appropriate clinical features, and supported by the demonstration of denervation of muscle on electrophysiological testing (4). Exclusion of other diseases, such as primary muscle disorders is sometimes required (5).

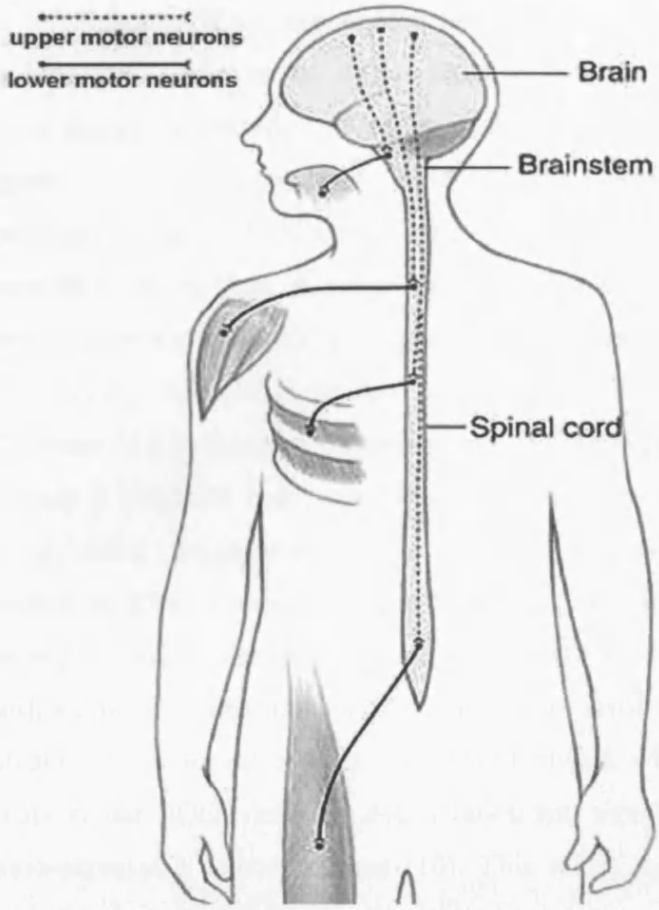


Figure 1.1- Upper and lower motor neurons

From The Muscular Dystrophy Association publications (1997) – "ALS Care"

ALS may present with bulbar involvement (swallowing and speech abnormalities), limb weakness, which can often be asymmetrical, or with the fasciculations that are seen in denervated muscles. Degeneration of the motor neurons can produce spasticity, wasting and weakness of the affected muscle group. Compromise of the motor supply to the muscles of respiration means that for many patients, respiratory failure is the eventual cause of death (2). Respiratory support, through artificial ventilation, may be employed with the aim of prolonging life, although this may be a difficult decision in the overall context of quality of life (6).

Pathologically, degeneration of the anterior horn cells within the spinal cord with subsequent distal motor neuron death occurs (1). The mechanism by which this process occurs is unknown, although a number of possible explanations have been suggested. Aberrant glutamate handling, with abnormal excitatory amino acid metabolism producing locally toxic agents has been suggested (7), and is considered in greater detail below (1.6). A subgroup of patients with familial ALS (FALS) have been shown to have a mutation in the superoxide dismutase 1 gene (SOD1) (see section 1.3). Protein aggregration within diseased motor neurons has been demonstrated in transgenic SOD1 mice to correspond to fragmentation of Golgi apparatus (8). The suggestion from this study is that there is a causal relationship between the aggregation of mutant SOD1 and ubiquitin, fragmentation of the Golgi apparatus of motor neurons and neurodegeneration. Oxidative damage mediated by free radical damage (9) has been proposed as a mechanism of cell death and is considered in greater detail in the context of mitochondrial abnormalities (1.5.2). The local involvement of non-neuronal cells in mediation of the disease process has been identified, with non-neuronal cells that do not express mutant SOD1 delaying degeneration and significantly extending the survival of mutant-expressing motor neurons (10). This raises the possibility that extra-neuronal mechanisms may be involved in the disease process.

The involvement of abnormal neuronal protein transport mechanisms in the pathogenesis of ALS was suggested by the demonstration of polymorphisms in the C-terminal region (11) and deletions in the tail (12) of the human neurofilament heavy protein gene in a limited number of individuals with ALS. This is involved in neuronal transport mechanisms and, therefore, potentially a number of different neuronal preocesses. A frameshift mutation in the peripherin (a neuronal intermediate filament associated with inclusion bodies) gene in a small number of patients with ALS supports the involvement of disordered transport mechanisms (13). The assembly of the micro-tubular elements of the neuronal transport system has been shown to be abnormal in SOD1 mice (14), and adds more weight to the aberrant transport hypothesis.

Although there are differing theories as to the cause of motor neuron death in ALS, it seems difficult to delineate discrete mechanisms (15).

At the present time, there is no curative treatment available for ALS. Riluzole is licenced for the treatment of the disease. It is thought to act by the inhibition of glutamate release (see 1.6) and may prolong life in patients with ALS for around 2 months when compared with a placebo (16).

1.1.1 Other Motor Neuron Diseases

ALS is a motor neuron disease with a clinical spectrum which includes primary lateral sclerosis (PLS) and progressive muscular atrophy (PMA). PLS is a purely upper motor neuron disease, PMA is a purely lower motor neuron disorder. There are rarer syndromes which may involve an ALS-type of clinical picture, with upper and lower motor neuron involvement, in association with more unusual features. Western Pacific ALS is a constellation of the classical motor neuron pattern of involvement seen in ALS with associated progressive dementia (17). The Parkinsonism-dementia complex found in Guam involves a disease phenotype which may contain elements of ALS along with Parkinsonian features and cognitive involvement (18). The genetic basis of this disease has yet to be elucidated (19). A combination of upper and lower motor neuron involvement with urinary incontinence, sensory disturbance and dementia is seen in polyglucosan body disease, due to the accumulation of PAS-positive polyglucosan bodies (PB) of various sizes and shapes in the cerebral hemispheres, brainstem, cerebellum, spinal cord, nerve roots and nerves (20). A syndrome of motor neuronopathy with cataracts and skeletal abnormalities has been described in a single 4-generation family (21). Although these diseases are relatively rare, when compared with ALS, their existence demonstrates that upper and lower motor neuron pathology can occur in conjunction within disease phenotypes other than ALS.

1.2 Mitochondria

Mitochondria are thought to have arisen from aerobic bacteria which colonised eukaryotic cells 10^8 years ago (22). This is known as the "endosymbiont hypothesis" and

proposes that the mitochondria that populate eukaryotic cells were once free-living bacteria that formed a symbiotic relationship with the eukaryotic progenitor cells about 20 x 10^8 years ago, when atmospheric oxygen concentration was increasing. These bacteria gradually lost their independence, becoming subservient to the nuclear genome. This is supported by the fact that mitochondria retain several "primitive" features that are also found in modern bacteria. However most of the original mitochondrial genome has long since been moved to the nuclear DNA. Mitochondria are dependent on numerous different proteins imported from the cytosol.

Mitochondria are composed of four compartments; an outer membrane, an inner membrane, the intermembrane space between the outer and inner membranes, and the matrix which is the region inside the inner membrane (23).

Mitochondria are organelles present in every cell in the body (including platelets). They are the major site of ATP production, through the five oxidative phosphorylation (OXPHOS) enzyme complexes located on the inner membrane of the mitochondrion. Mitochondria have their own genome. Mitochondrial DNA (mtDNA) is a double-stranded DNA molecule of 16,569 base pairs (figure 1.2). This encodes 13 polypeptides of the OXPHOS system, with the remaining 73 polypeptides being encoded by the cell nucleus (figure 1.3) (24). Components of complex I (NADH ubiquinone reductase), III (ubiquinol cytochrome c reductase), IV (cytochrome c oxidase) and V (ATP synthase) are encoded for by the mtDNA, whereas complex II (succinate ubiquinone reductase) is entirely encoded by the nuclear DNA. Differences in base excision repair mechanisms mean that mtDNA is more vulnerable to oxidative stress than nuclear DNA (25). It is also thought that the proximity of the mtDNA molecule to the generative sites of reactive oxygen species (through the mitochondrial electron transfer system) means that the mtDNA is more vulnerable to damage from free radicals than nuclear DNA (26), which is located some distance from the point of ATP generation.

Cells contain many thousands of copies of the mtDNA molecule. A mutation of the mtDNA molecule may be present in only a portion of the mtDNA molecules present in any one cell. This is important in mitochondrial disease where disease expression only comes about where a threshold of mutated mtDNA is reached (see 1.2.1).

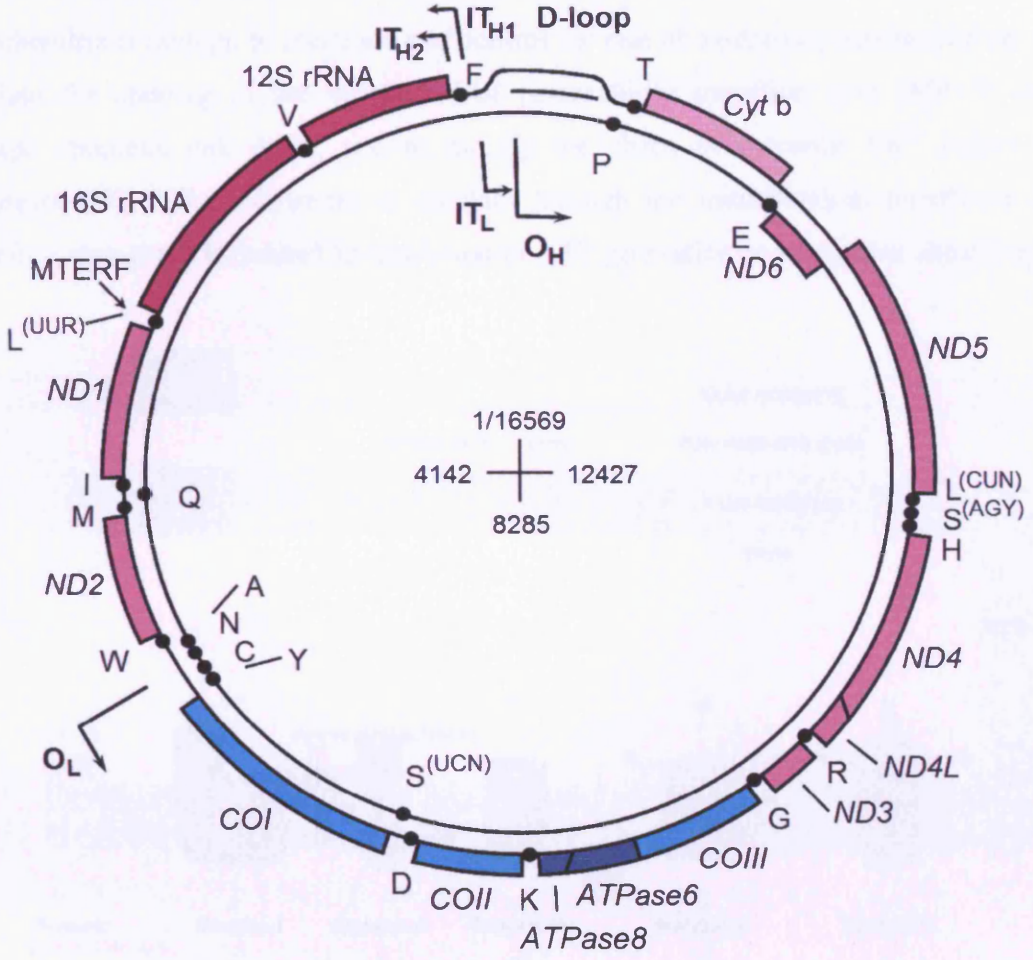


Figure 1.2 The mtDNA molecule showing encoding regions for the different proteins. The outer circle represents the heavy (H) strand – it's origin of replication (O_H) is shown as is the origin of replication (O_L) of the inner, light strand. ND1, 2, 3, 4, 4L, 5 and 6 are the genes ecoding subunits of NADH dehydrogenase; COI, II, III encode subunits of cytochrome c oxidase; cyt b encodes the cytochrome b component of ubiquinol cytochrome c reductase; MTERF=Mitochondrial transcription termination factor; ATPase6 and ATPase 8 are subunits of complex V (ATP synthase). The tRNA genes are shown as black dots and are labelled with the single character for the Amino Acid the tRNA picks up and delivers to the Ribosome during protein synthesis (courtesy of Dr JW Taanman).

Apart from energy production and attendant free radical generation, the mitochondria have a role in intracellular calcium homeostasis. Calcium sequestration by the mitochondria is thought to stimulate and control the rate of oxidative phosphorylation, to regulate the opening of the mitochondrial permeability transition pore (MPTP) and perhaps apoptotic cell death, and to modify the shape of cytosolic Ca^{2+} pulses or transients (27) . The movement of calcium through the mitochondrial membrane is, therefore, thought to be related to regulation of ATP generation and apoptosis signaling.

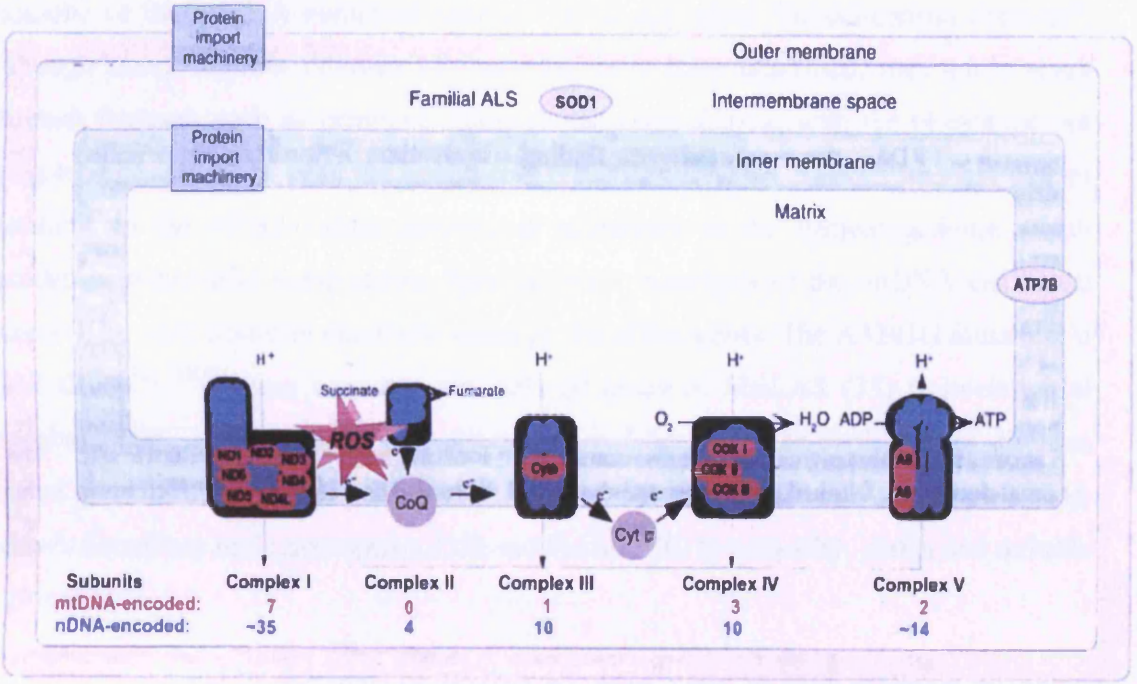


Figure 1.3 The mitochondrial respiratory chain complexes I-V on the inner mitochondrial membrane demonstrating the electron transfer system (complexes I to IV), the proton cycle and the contribution of mtDNA- and nuclear DNA-encoded components (shown in red and blue, repectively) to each complex. CoQ is co-enzyme Q; cyt c is cytochrome c. The generation of reactive oxygen species (ROS) resulting from electron transfer is shown. ATP7B is ATPase, Cu^{2+} transporting, beta polypeptide.

From: Schon, E.A., Manfredi, G. 2003. Neuronal Degeneration and Mitochondrial Dysfunction. *J. Clin. Invest.* **111**:303-312.

MtDNA is transmitted through the female germ line (28). Although a single case of partial paternal transmission has been reported (29), inheritance of paternal mtDNA is considered exceptionally rare (30,31). Therefore, diseases associated with with mtDNA mutations generally display maternal inheritance.

1.2.1 Mitochondrial Diseases

There are a number of disorders associated with abnormalities of the mitochondrial organelle or the mtDNA contained therein. These are called "mitochondrial diseases". Although many discrete diseases of this type have been described, they often share common features, such as deafness, seizures and short stature, with the phenotype not being readily associated with the genotype (32). Mitochondrial diseases may arise from mutations in the mitochondrial genome or mutations in the nuclear genome which encodes mitochondrial components. Specific point mutations of the mtDNA can cause disease. The most common mutations occur in the tRNA genes. The A3243G mutation in the 3 $tRNA^{leu\ (UUR)}$ gene accounts for 80% of cases of MELAS (33) (mitochondrial encephalopathy, lactic acidosis and stroke-like episodes), for example. Other diseases caused by mtDNA point mutations include maternally inherited Leigh's syndrome (34), Leber's hereditary optic neuropathy (35) and NARP (36) (neuropathy, ataxia and retinitis pigmentosa).

Diseases caused by deletions of mtDNA are usually due to large deletions involving both tRNA and protein encoding mitochondrial genes. Disorders caused by single deletions of mtDNA include Kearns-Sayre syndrome (37) and sporadic ocular myopathy (38). Multiple mtDNA deletions are found in patients with inclusion body myositis (39) and autosomal dominant progressive external ophthalmoplegia (40).

Mitochondrial genetic disorders may demonstrate a phenomenon whereby the phenotype changes when the threshold of mtDNA abnormalities in a previously unaffected tissue is surpassed (41). The threshold for the development of disease is lower in tissues highly dependent on oxidative metabolism such as the brain, heart, skeletal muscle, retina, renal tubules and endocrine glands.

A mutation in one mitochondrial DNA molecule within a cell leads to a mixture of mutant and normal mtDNA molecules - this is termed heteroplasmy During mitosis it is a matter of chance which mtDNAs will be passed to each daughter cell.

Over many cell divisions the proportion of normal and mutant mtDNA molecules can drift towards either pure mutant or normal (homoplasmy). This can occur during the replication of somatic cells or during proliferation of female germinal cells. Hence the threshold for the development of a mtDNA disorder may be said to be reached when the mutant load of mtDNA copies is sufficiently high.

With ageing, there is an accumulation of somatic mtDNA mutations (42). It is hypothesised that this process produces alterations in the expression of the mitochondrial genome which may produce some of the "pathological" changes associated with the ageing process (43) . A study looking at mtDNA analysis and respiratory chain function assessment in muscle (44) has demonstrated that increasing age produced deficiencies in respiratory chain function and increasing amounts of mtDNA deletions in some individuals. This study failed to correlate the enzyme activities and mtDNA deletions, directly, however, and could not prove that decline in respiratory chain function was due to mtDNA alterations, alone. A mouse model, utilising increased levels of mtDNA mutations through a proof reading deficiency, has been shown to develop early ageing in association with increased markers of apoptotic activity (45), but not an increase in markers of oxidative stress.

1.3 Familial and Sporadic ALS

Approximately 10% of all patients with ALS have a positive family history of the disease (46), although the majority of cases are sporadic. Mutations of the SOD1 gene, which encodes the enzyme copper/zinc superoxide dismutase, are found in approximately 20% of familial patients (47). SOD1 mutations are found in 1-2% of all patients with ALS (ALS1) (48). The SOD1 gene is on the long arm of chromosome 21 (21q22.1). Many different mutations with predominantly dominant inheritance have been found in

the 5 exons of this gene (49). No mutations coding for SOD2, a closely related compound which localizes to the mitochondria have been found in familial or sporadic ALS (FALS and SALS, respectively) (50). Mutations in the SOD1 gene have also been identified in a proven sporadic case of ALS (51), in apparently sporadic cases (46) as well as with recessive inheritance (52).

Other, rarer, genetic abnormalities have been detected in patients with ALS, including the alsin (53) and APEX (54) genes, but the significance of some of these is uncertain at the present time. Other genes have been identified which have been linked with particular phenotypes or with an increased susceptibility to the development of ALS (Table 1.1).

At the current time the vast majority of research into the pathogenesis of ALS has focused on SOD1 transgenic mice (see 1.4.1 below) and cell models, as these are felt to provide a good disease model of both FALS and SALS (55). Less work has been performed in comparing familial with non-familial, or SOD1 with non-SOD1 patients. Work on fibroblast cultures, showing differing sensitivities to oxidative stress in SALS and FALS (56) demonstrated that cells from sporadic patients were much more sensitive to oxidative stress than SOD1 FALS fibroblasts. Indeed, using serum withdrawal as a stressor induced no difference in stress between control and FALS SOD1 fibroblasts. Post mortem central nervous system tissue showed respiratory chain enzyme activity differing in the brains of SALS and FALS patients (57), with reduced SOD1 activity and increased complex I activity in FALS-1 patients and increased oxidative damage to proteins in SALS patients. These studies suggest that there may well be differences between the disease types in terms of cellular pathology, especially as regards possible mitochondrial involvement. There is great heterogeneity in phenotypic presentation both within and between the different SOD1 genotypes, and even more between the other genes that have been identified as causing ALS. This may suggest that particular transgenic mouse or cell models may only be good models for the genotypes involved, with a limited application to sporadic, or other familial forms.

Table 1.1 The current known causative and susceptibility genes for ALS.

Name	Gene / locus	Epidemiology	Inheritance	Age Onset (years)	Clinical Features
ALS1 (47)	SOD1 21q	10% of familial cases	Usually Dominant	Adult (mean=46)	Similar to SALS, homozygotes more severe phenotype.
ALS2 (58)	Alsin 2q33	10 known families	Recessive	Childhood (mean=6.5)	Spasticity, very slowly progressive, no sensory or bladder involvement.
ALS3 (59)	18q21	Large French family	Dominant	Adult (Mean=45)	Upper and lower neuron involvement, mean progression=5 years.
ALS4 (60)	Senataxin 9q34	England and Maryland	Dominant	2nd decade	Early gait disorder and distal weakness, slowly progressive.
ALS5 (61)	15q15	Europe and North Africa	Recessive	Teens	Predominantly lower motor neuron distal pattern of involvement.
ALS6 (62)	16q	4 known families	Dominant	Mean 38 to 67 years	Generalised involvent with variable progression.
ALS7 (62)	20p13	1 family	Dominant	Adult (mean=57)	Progression over 3 years.
ALS8 (63)	VAPB 20q13.33	7 Brazillian families	Dominant	Adult (mean=35)	Heterogenous presentation between cases with variable progression.
ALSX	Xp11-q12	1 family	Dominant	Adult, 40 - 60	Bulbar dysfunction, slowly progressive.
Neurofilament heavy chain (11)	22q12	5 patients	Dominant or sporadic	Variable	Variable, may involve dementia and bulbar symptoms.
APEX (54)	14q11.2-12	4 patients	Sporadic		None consistent.
Peripherin (64)	12q12-q13	2 Patients	Sporadic		Asymetrical leg weakness, progresses over 5 years.

One of the main difficulties that has been encountered in the study of the pathogenesis of SALS is that of trying to identify discrete phenotypes which may be of value in proteomic analysis (65) in an attempt to elucidate specific genetic causes of the disease.

The inheritance patterns of familial ALS cases have been analysed, and examples of autosomal dominant, autosomal recessive and X-linked kindreds have all been described (66), indicating that there are likely to be many potential genetic aetiologies.

1.4 Superoxide Dismutase

Of those patients with familial ALS, 5-10% have identifiable mutations in the gene which codes for superoxide dismutase 1 (SOD1) (47,66,67). SOD1 is an enzyme which removes reactive superoxide radicals ($O_2^{\cdot -}$) from cells by converting them to a harmless form ($2H^+ + O_2^{\cdot -} \longrightarrow H_2O_2 + O_2$) The superoxide radicals, which are by products of normal cellular processes, can damage cells if their levels are not controlled by superoxide dismutase. The active form of SOD1 incorporates a zinc and copper ion into its 3-dimensional structure. Although primarily a cytosolic enzyme, it has been posited that a portion of the SOD1 localises to the intermembrane space of the mitochondrion with a metallochaperone, which protects the cell from mitochondrial oxidative damage (68). Subsequent work has questioned whether the toxic effect exerted by the mutant SOD1 is dependent upon copper handling abnormalities (69), demonstrated by ablating the gene encoding the copper chaperone for SOD1 (CCS) in a series of FALS-linked SOD1 mutant mice, and showing that this did not modify the onset and progression of motor neuron disease in SOD1-mutant mice. Using dorfin, a ligase for mutant SOD1, accumulation of the mutant SOD1 in mitochondria was significantly reduced in a neuronal cell model, with resulting inhibition of cytochrome c release and caspase activation, thus reducing neuronal cell death (70). This would imply that inhibition of the toxic properties of mutant SOD1 would have a protective role for the cell.

Crystal structure studies of mutant SOD1, using X-ray diffusion techniques have shown that mutations in the amino acid sequence at the surface of the molecule may cause significant conformational change and destabilisation of the enzyme (figure 1.4) (71). This would imply that point mutations in the sequence could produce a toxic gain of function through alterations in binding of the SOD1 molecule.

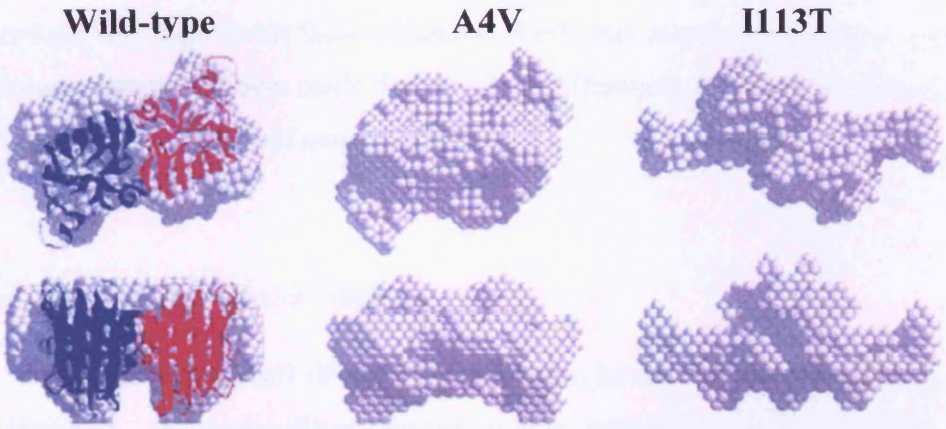


Figure 1.4 Crystallographic scatter studies of wild type and 2 mutant (A4V and I113T) SOD1 molecules demonstrating conformational surface changes (72).

1.4.1 Transgenic SOD1 Mice

The first SOD1 transgenic mice, carrying the G93A mutation, which is sometimes carried by FALS patients, were developed in 1994 (73). The actual site of the SOD1 G93A transgene insertion site lies on distal mouse chromosome 12 (74). These mice become paralysed in one or more limbs and die by 4-5 months of age. It has been shown that the presence of the SOD1 mutation produces a toxic gain in function. These mice develop degeneration of the motor neurons. Extensive pathology develops within the spinal cord of these mice some time prior to the onset of clinically apparent disease. Golgi apparatus fragmentation precedes microvacuolar changes in the mitochondria of the spinal cord, with swelling of the mitochondrial intermembrane space. Further analysis of these mice revealed that ubiquinated cytoplasmic inclusion bodies containing

the mutant SOD1 develop (75). These changes bear strong similarities to the pathological changes seen in the post mortem spinal cords of patients with ALS. The mutant SOD has been shown to colocalise to the mitochondria in the central nervous sytem of transgenic mice (76). Many therapeutic trials have been performed using the SOD1 mouse model and various neuroprotective agents, none have, as yet, translated into appropriate treatments for the human ALS disease (77).

To develop pathological changes, these mouse models over-express mutant SOD1 to produce very high levels intra-cellularly. While this may be important in manifesting the disease phenotype, there could be important differences between the disease mechanisms in the human disease and mouse models.

1.4.2 SOD1 Neuronal Cell Models

Neuronal cell models of the SOD1 mutation have been developed. Using micro-array techniques, an immortalized mouse motor neuronal cell line NSC34 was stably transfected with either normal or mutant (G37R, G93A, I113T) SOD1 cDNAs, and the effect of the presence of these proteins on gene expression was analysed (78). These experiments have demonstrated that the presence of the mutant SOD1 leads to a decrease in expression of *kif*3b, a kinesin-like protein, which forms part of the kif3 molecular motor. The expression of c-*fes*, a cytoplasmic protein-tyrosine kinase thought to be involved in intracellular vesicle transport was also decreased. In addition, a decrease in expression was confirmed for intracellular adhesion molecule 1 (ICAM1) in the presence of all three of the mutations studied (78). Further work with this cell model has demonstrated the development of ultrastructural mitochondrial abnormalities and diminished activity of respiratory chain complexes III and IV (79) in the presence of mutant SOD1. Molecular studies of this neuronal cell model of ALS have suggested that the mutant SOD1 may exert a toxic effect through the generation of reactive oxygen species (ROS) as well as direct inhibition of mitochondrial function (80). More recent work on the G93A neuronal cell model (81) using gene expression profiling, showed that a number of DNA transcripts were differentially expressed in the presence of mutant

human SOD1, including the antioxidant response element genes encoding certain detoxifying enzymes and antioxidant response proteins.

Glutamate transport in a G93A human neuroblastoma SHSY-5Y cell model was more sensitive to oxidative stress as induced by growing cells in media supplemented with 0.3 mM paraquat (82) than in non-transfected cells. This study did not show an attenuating effect of anti-oxidant treatment to the effects of the paraquat on glutamate uptake by the cell cultures.

There is evidence that the surrounding environment of non-neuronal cells can influence the toxicity of mutant SOD1 expressed in motor neurons; in chimeric transgenic SOD1 mice, the survival of SOD1 motor neurons was extended by the presence of wild type non-neuronal cells (83). This would suggest that while mutant SOD1 cell models can provide valuable information about changes gene expression, they may be less useful in modeling the disease state per se.

1.5 Evidence for the Involvement of Mitochondria in the Pathogenesis of ALS

1.5.1 Structural Studies

Electron microscopy of human motor neurons from patients with ALS has revealed similar mitochondrial abnormalities to those developing in SOD1 mutant mice. For example, mitochondrial structural abnormalities have been identified in the post mortem corticospinal tract (84) (axonal swellings were seen to consist of accumulations of neurofilaments and altered mitochondria) and ventral horn cells (85) (scattered mitochondria, vesicles and fragments of smooth endoplasmic reticulum were found among interwoven bundles of neurofilaments) of those affected. The mitochondria in each case were swollen with disrupted membrane abnormalities evident. Ultrastructural studies on remote tissues have shown similar patterns of mitochondrial abnormalities in patients with ALS, including the liver (86) and motor end plates (figure 1.6) (87).

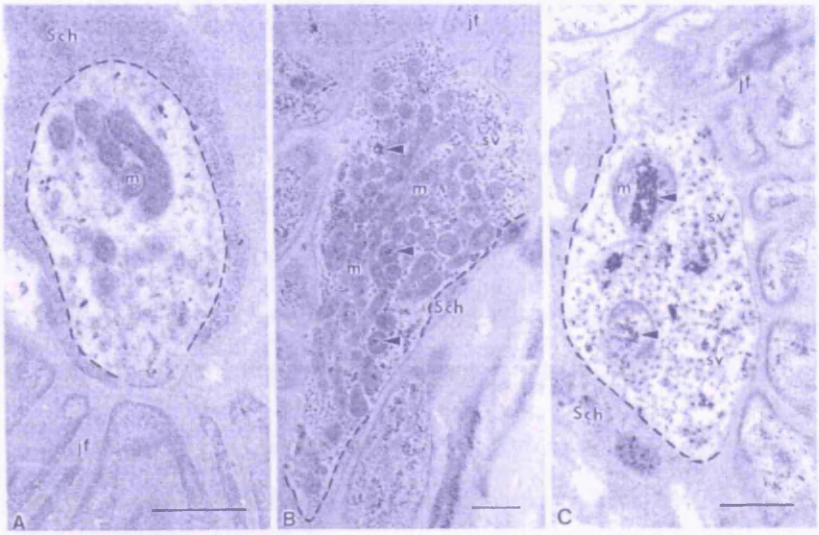


Figure 1.6 Motor end plates from a patient with a peripheral neuropathy (disease control) (A) and 2 patients with ALS (B,C) show a higher density of larger mitochondria in the ALS patient samples. Sch = Schwann cell envelope, m = mitochondria, sv = synaptic vesicles, jf = junctional folds. Calcium precipitates are arrowed. Bar = 0.5 μm
(From: Siklos et al. Ultrastructural evidence for altered calcium in motor nerve terminals in amyotrophic lateral sclerosis. Ann Neurol.1996;39:203-16).

1.5.2 Mitochondrial Mediation of Oxidative Damage

The role of mitochondria in the generation and homeostasis of free radicals within the cell, leads to the hypothesis that an abnormality of mitochondrial function will lead to oxidative damage through the action of ROS (88). Accordingly, markers of oxidative damage have been found to be increased in the brain (57) and spinal cord (89) of patients with ALS. Levels of oxidative damage have been shown to be elevated in the central nervous system DNA of the SOD1 G93A transgenic mouse model of ALS (90). A mitochondrial uncoupling protein (MUCP3) has been shown to be upregulated in the muscle of SOD1 transgenic mice as well as the muscle of patients with sporadic ALS, but not in the spinal cord (91). MUCP are thought to have a role in the regulation of mitochondrial activity in situ, and changes in levels of expression may represent changes in intracellular mitochondrial function. These findings suggest that while the primary site

of damage in ALS is the motor neurons, a more generalised metabolic abnormality may be implicated in the pathogenesis, or as a secondary effect.

Analysis of the central nervous system of transgenic SOD1 mice has also revealed increased levels of oxidative damage to DNA as measured by 8-hydroxy-2-deoxyguanosine (8-OHdG) (92). Staining for the immunoreactivity of 8-OHdG was not localized in the nucleus but in cytoplasm with small granular pattern, which may suggest that an oxidative damage to mtDNA is occuring in these mice.

A large prospective study of individuals using vitamin supplements identified that regular users of vitamin E, which acts as cellular antioxidant, were at a lower risk of dying of ALS than the population as a whole, including individuals taking vitamin C supplements or multi vitamin preparations (93). Although this study was controlled for age and smoking status, it was not blinded, and relied totally on self reporting.

1.5.3 Calcium Homeostasis

Abnormalities in the cellular homeostasis of calcium have been demonstrated in patients with ALS. Homogenised muscle biopsy samples of patients with ALS demonstrate elevated mitochondrial calcium levels (87). Peripheral blood lymphocytes show increased cytosolic calcium levels and impaired response (an increase in basal oxygen consumption rate) to inhibitors of oxidative phosphorylation (94). In a neuronal cell line expressing mutant SOD1, mitochondrial membrane potential was shown to be diminished and cytosolic calcium levels increased (95). Increased mitochondrial Ca^{2+} concentrations as a result of excitotoxicity (as for highly metabolically active cells such as neurons) has been associated with the generation of superoxide and may induce the release of proapoptotic mitochondrial proteins, proceeding through DNA fragmentation/condensation and culminating in cell demise by apoptosis and/or necrosis (96). This process has been implicated in the pathogenesis of many neurodegenerative diseases.

1.5.4 Mitochondrial Enzyme Activity

The main role of mitochondria is energy production which is achieved through the generation of ATP through the oxidative phosphorylation complexes (I – V) on the inner mitochondrial membrane (24) (figure 1.1). Direct assessment of mitochondrial function may be demonstrated through assaying the activity of these complexes. For example, a specific decrease in complex IV has been described in the spinal cord (97), muscle (98) and anterior horn cells (9) of patients with ALS. Obviously the central nervous system assays were performed on post mortem tissue. Corresponding complex II activity in the anterior horn cells was found to be normal (9). This finding is important, as complex II is entirely coded for by nuclear DNA, while complex IV is partly coded for by mtDNA. A single patient with an ALS type disorder has been described with a severe muscle complex IV deficiency due to an out of frame mutation in the mitochondrial gene for the major subunit of complex IV (99). G93A mice, transgenic for an SOD1 mutation associated with familial ALS, show evidence of generalised mitochondrial respiratory chain deficiency with impaired ATP synthesis in central nervous system tissue (100,101).

A neuronal SOD1 transgenic cell model was investigated for respiratory chain complex activity and cell death under oxidative stress. Complex II and IV activity was found to be diminished, and serum withdrawal from the growth medium, to generate oxidative stress, produced increased levels of cell death in the model relative to wild type SOD1 controls (102).

Although there is evidence of mitochondrial electron transfer chain abnormalities, the findings are not uniformly consistent between human tissue, mouse models and cell models. For example, complex I levels were found to be low (103) or normal (104) in the skeletal muscle of patients with SALS, increased in the brains of patients with FALS (105), low in the spines of transgenic SOD1 mice (101) and normal in a transgenic SOD1 cell model (79). Although variations in respiratory chain enzyme activities may point to the involvement of mitochondria in the pathogenesis of ALS, a clearly defined relationship has yet to be proven.

1.5.5 MtDNA Mutations

A wide range of degenerative conditions have been described in association with mtDNA (mtDNA) mutations (106). For instance, mtDNA abnormalities in association with electron transport chain deficiencies (107) have been described in neurodegenerative diseases such as Parkinson's disease (108,109) and nuclear genetic abnormalities coding for mitochondrial proteins have been described in Huntington's disease (110) and Friedreich's ataxia (111).

Although abnormalities of mtDNA have been documented in ALS patients in some reports, other reports found no evidence of such an association. In one study, decreased levels of mtDNA have been demonstrated in the muscle of 14 out of 17 patients with ALS (112). The absolute number of mitochondria was unaffected, as illustrated by the normal citrate synthase levels within the tissue concerned. Low levels of mtDNA were also found in the brains of patients with ALS (113). This study also measured levels of the 4977 base pair deletion of mtDNA. In patients with ALS, levels of the deletion were higher in the motor cortex than the temporal cortex in patients with ALS compared with normal controls. The 4977 base pair deletion has previously been shown to be elevated in the normal aged brain (114). Levels of this mutation have recently also been found to be elevated in skeletal muscle from a proportion of patients with ALS compared with the levels found in patients with other neuromuscular disorders (115) This would implicate mtDNA degeneration as a possible factor in the pathogenesis of SALS.

In contrast to the above studies which suggest the association of ALS with mtDNA abnormalities, single cell analysis of post mortem tissue demonstrated no difference in the levels of the common deletion of mtDNA in the motor neurons of patients with SALS and controls (116), The finding would suggest that significant accumulation of mtDNA deletions in the central nervous system is not the cause of SALS. This is supported by a study comparing mtDNA levels in the skeletal muscle of patients with SALS and neurogenic controls with normal controls (117). The study identified that there was only a minimal decline of mtDNA:nDNA ratios in SALS and disease control patients in comparison with healthy controls.

Nevertheless, in some patients, specific mtDNA mutations have been identified that are considered to be involved in the pathogenesis of the disease. As mentioned above (1.6.4), a single patient with ALS has been described with low levels of muscle complex IV activity occuring secondary to a mtDNA mutation causing a deficiency in the expression of a subunit of complex IV (99). Furthermore, a single patient with a motor neuron disease associated with temporal lobe epilepsy was found to have a mutation in the mitochondrial tRNA gene (118). This was associated with mild complex I deficiency in skeletal muscle. Even so, the mutation was not identified in a cohort of 40 patients with SALS. The association of a disorder of the motor neurons with a mtDNA mutation and evidence of respiratory chain deficit in remote tissues supports the possibility of mtDNA abnormalities in the pathogenesis of SALS.

1.5.6 Cybrid Studies and Platelets

An important technique for determining whether mitochondrial or nuclear DNA is the source of observed pathological cellular abnormalities is provided by cybrid studies. These involve the fusion of patient platelets (which contain mtDNA, but do not have a nucleus, and therefore lack nuclear DNA) with experimental ρ^0 cells. ρ^0 cells are cells that have been depleted of mtDNA, but contain nuclear DNA. The fusion results in the patient mitochondria being presented in a control nuclear context (figure 1.7). Early work with cybrids, indicated a deficiency in platelet complex I activity of patients with Parkinson's disease (119). A later study of cybrids derived from the platelets of ALS patients revealed several deficits in the cell line that were not present when control platelets were fused with the SH-SY5Yρ^0 cells (120). These included a decrease in complex I activity as well as an increase in free radical scavenging activity and abnormalities of calcium homeostasis, similar to those described above. Other work performed at a later time (121) suggests that citrate synthase, glutamate dehydrogenase and and cytochrome oxidase activities are the same in platelets from patients with sporadic ALS and controls. More recent work suggests that the repopulation of 143B ρ^0 cells with mtDNA from the platelets of patients with ALS restores normal respiratory

activity within those cells (122). Although different recipient cells were used in each case, respiration in intact SH-SY5Y and 143B cells has been shown to be similar (123). SH-SY5Y cells are a line derived from human neuroblastoma cells, while 143B cells are derived from a human osteosarcoma line (124). The differences in results may well be explained by the differences in experimental conditions between the two studies, rather than proving or disproving the existence of a mitochondrial abnormality in the platelets of patients with ALS.

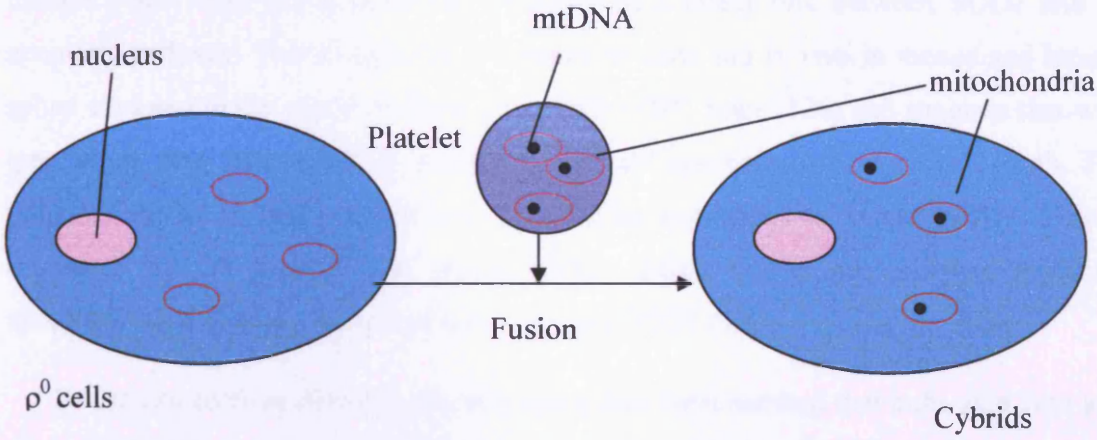


Figure 1.7 Fusion of anuclear platelets with ρ^0 cells lacking mtDNA. The donor mtDNA is obtained from platelets, and repopulates experimental cybrid cells.

1.5.7 SOD1 and Mitochondria

A subcellular fractionation study of transgenic G93A mice and a single SALS patient (72) demonstrated the presence of mutant SOD1 in the spinal cord mitochondria, whereas virtually no wild type SOD1 localised to these organelles. Recruitment of abnormal SOD1 occurred independently of copper chaperone or dismutase activity. A highly preferential association with spinal cord mitochondria in human ALS for a mutant SOD1 that accumulates only to trace cytoplasmic levels (72) has been shown. This provides a link between the observed mitochondrial abnormalities detailed above, and the

production of disease causing SOD1 mutants in FALS. In a more recent study, it was demonstrated that, in addition to being in the mitochondrial outer membrane and intermembrane space, mutant SOD1 is also localised to the matrix (125). Aberrant SOD1 macromolecular aggregates were found in the matrix of brain mitochondria, suggesting that mutant SOD1 in the brain mitochondrial matrix is misfolded and prone to aggregation. This may contribute to selective neuronal degeneration.

Further work has been performed demonstrating that the mutant SOD1 molecule binds to bcl-2, a mitochondrial anti apoptotic protein (126). While the wild-type protein is anti-apoptotic, mutant SOD1 promotes apoptosis. It was shown that both wild-type and mutant SOD1 bind bcl-2, providing evidence of a direct link between SOD1 and an apoptotic pathway. This interaction is evident *in vitro* and *in vivo* in mouse and human spinal cord and in the spinal cords of transgenic SOD1 mice (126) and suggests that wild type SOD1 may have a role in protecting the cell against programmed cell death. The proposed model involves the binding of bcl-2 by aggregates of mutant SOD1. This is supported by the finding that mutant SOD1 linked to the mitochondria promotes apoptosis more strongly than a cytososlic mutant SOD1 (127).

In contrast to these findings, another group has demonstrated that both wild type and mutant SOD1 are to be found in the intermembrane space of SOD1 G93A transgenic mice overexpressing mutant human SOD1 (100). This study identified evidence of morphological and functional abnormalities in mitochondria from the spinal cord of affected animals, including oxidative damage to mitochondrial proteins and lipids and deficiencies in the electron transfer chain, and ATP synthesis.

1.6 The Glutamate Hypothesis

Glutamate is the major excitatory neurotransmitter within the central nervous system. There are a number of different subtypes of glutamate receptors distributed thoughout the CNS. Abnormal activation of these receptors has been associated with neuronal death through the activation of a number of pro-apoptotic emzymatic pathways (128).

Disturbances in calcium homeostasis and free radical mediation of neurotoxicity are also thought to depend on aberrant glutaminergic neurotransmission. (129).

Glutamate levels are found to be elevated in the serum (a mean increase of 100% in patients compared with controls) (130) and the CSF (a mean increase of 100 – 200% in patients compared with controls) (131) of patients with sporadic ALS. The involvement of glutamate transmission in the pathogenesis of the disease is further supported by the use of riluzole, a glutamate release inhibitor, in slowing the progression of the disease. Riluzole has been shown to have a modest effect in prolonging survival in ALS (132). Glutamate toxicity can occur both acutely, and in a chronic, irreversible, manner. (133) This would mirror the progressive nature of the disease in ALS patients.

In studies of another SOD1 transgenic model, cultured motor neurons from the G93A SOD1 transgenic rat showed increased sensitivity to the excitotoxic effects of glutamate relative to controls (134). These effects were attenuated by the antioxidant vitamin E, and survival time was increased in the animals by the use of riluzole and gabapentin, both glutmatate antagonists. Unlike riluzole, gabapentin has not been shown to be of therapeutic benefit in human ALS (135).

With regard to the role of mitochondria in glutamate toxicity, neuronal cells which are overstimulated by glutamate undergo excess calcium influx. Blocking the uptake of calcium by the mitochondria significantly reduced glutamate toxicity (136). This would imply that perturbed mitochondrial function results in changes in the flux of calcium through the cell which may mediate neurotoxicity by glutamate receptor activation.

1.7 Hypothesis

Following review of the pathogenesis of ALS and the mitochondrial pathology, as described above, the following hypothesis was proposed and tested in the series of experiments which are described:

Cumulative mtDNA (as opposed to nuclear DNA) mutations resulting from oxidative damage in mitochondria contribute to motor neuron death and the development of motor

neuron disease. These mutations are random with a threshold effect that would explain the selective late onset motor neuron death. Motor neurons are especially vulnerable to oxidative damage due to their structure and high metabolic turn over.

During the performing of these experiments, a further hypothesis was developed:

ALS is the result of a systemic hypersensitivity to oxidative stress.

2 Methods and Materials

2.1 Patients and Controls

2.1.1 Patient information and Consent

Patients and control subjects were recruited from neurology outpatient clinics. Control subjects were also recruited from inpatients at the Royal Free Hospital who were undergoing muscle biopsy for other diagnostic purposes. Each subject was given a comprehensive information sheet and a full verbal explaination of the study was provided, with opportunities to discuss any issues arising or any questions that the subject may have had. Ethical approval was obtained from the Royal Free Hospital Local Research Ethics Committee.

Informed consent was obtained, seperately, for the muscle, blood (platelets) and skin samples. A record was made of the level of power in the limb that was being biopsied for the study. All patients had MRC grade power of 3 or greater.

Because all of the patients and controls had a muscle biopsy performed for diagnostic purposes, these biopsies were examined to ensure that "normal" controls did not, in fact, have demonstratable pathology on microscopy. Normal controls had a final clinical diagnosis of no muscle or other physical disease. The samples were also scrutinised to ensure that patients who fulfilled the diagnostic criteria for motor neuron disease did not have other muscle pathology.

None of the patients or controls smoked at the time of the study. Past smoking history was not recorded.

2.1.2 Patients

There were 12 ALS patients enrolled in the study. Mean age was 57.3 years (range 33-70 years). The mean time from diagnosis was 5.25 years. (table 2.1) MRC Power score in the affected limb ranged from 3 to 5. At the time of writing, as far as can be ascertained, all of the patients in the study are still alive. One patient has familial ALS with an SOD1 mutation (figure 2.1) (see section 3). ALS was defined by the El Escorial criteria for the diagnosis (137).

Table 2.1 Patients

Name	Sex	Diagnosis	Power in biopsied limb	Age at Study	Time from Diagnosis (years)
PA1	M	ALS	5	33	2
PA2	M	ALS	5	61	8
PA3	F	ALS	4	56	4
PA4	M	ALS	4	68	11
PA5	M	ALS	3	70	18
PA6	F	ALS	3	43	4
PA7	F	ALS	4	60	1
PA8	M	ALS	5	57	2
PA9	M	ALS	3	58	7
PA10	M	ALS	3	53	1
PA11	F	FALS	4	65	4
PA12	M	ALS	4	64	1

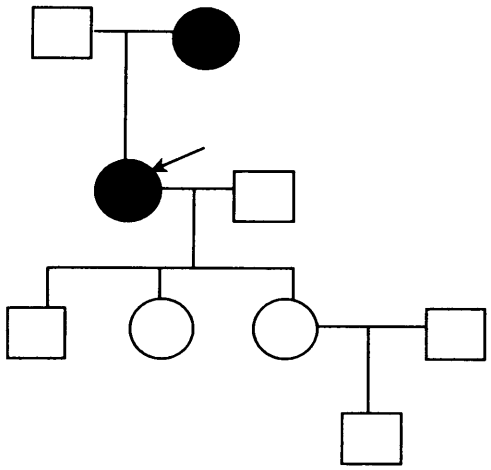


Figure 2.1 Pedigree for PA11 (arrowed), patient with familial ALS. Filled figures are affected individuals. The pedigree shown is incomplete for reasons of confidentiality.

2.1.3 Disease Controls

There were 11 disease controls recruited to the study, with a mean age of 55.3 years (range 18-71 years). These had peripheral neuropathies, benign fasciculations and hereditary spastic paraplegia (HSP) (table 2.2). Subsequent studies (138) demonstrated complex I respiratory chain enzyme mitochondrial abnormalities in the patients with HSP and, therefore, they were withdrawn from the study.

Table 2.2 Disease Controls

Name	Sex	Diagnosis	Age at Study
D1	M	Motor Neuropathy	54
D3	M	Peripheral Neuropathy	59
D4	M	Benign Fasciculations	66
D5	M	Peripheral Neuropathy	67
D6	M	Peripheral Neuropathy	69
D7	F	Progressive Weakness Unknown Cause	18
D8	M	Benign Fasciculations	39
D9	F	HSP	54
D10	M	HSP	44
D11	M	Benign Fasciculations	67
D12	M	Post Polio Syndrome	71

2.1.4 Controls

Twenty one patients were recruited as controls. They had a mean age of 47.9 years (range 26-82 years). These patients had a range of non-specific symptom defined conditions that were being investigated at the Department of Neurology, Royal Free Hospital. All of the controls had normal muscle biopsy histology and no evidence of neurological disease clinically. This group was not individually age- and sex- matched, but had a similar age range to the patient and disease control groups.

Table 2.3 "Normal" controls

Name	Sex	Potential Diagnosis (reason referred for muscle biopsy)	Age at Study
C1	F	Myalgia	26
C2	F	Myalgia	27
C3	F	Weakness	60
C4	M	? Polymyositis	73
C5	M	Myalgia	45
C6	M	Myalgia	27
C7	M	?Myasthenia – not proven	79
C8	F	Muscle weakness and learning difficulties	56
C9	F	Proximal Weakness	82
C10	F	Proximal Weakness	60
C11	M	Muscle Weakness	20
C12	M	Myalgia	37
C13	F	Weakness	38
C14	F	Generalised weakness	61
C15	F	?Dermatomyositis	44
C16	F	?Myasthenia – not proven	31
C17	F	?Myalgic encephalomyelitis	30
C18	M	Chronic fibrosing alveolitis	61
C19	F	?Myalgic encephalomyelitis	52
C20	M	Myalgia	51
C21	F	Generalised weakness	45

2.2 Materials

2.2.1 Equipment

Unless specified otherwise, the following equipment was used.

Tissue Culture – ICN-Flow Automatic CO_2 Incubator Model 320 (ICN-Flow Ltd., High Wycombe, Bucks, UK). Envair Class II Microbiological Safety Unidirectional Laminar Cabinet (Envair Ltd., Rossendale Lancashire, UK).

Homogenisation – Uni-form 10ml glass/teflon homogeniser (Jencons Ltd., Leighton Buzzard, Bedfordshire, UK).

Spectrophotometers – Hitachi U-3220 and Hitachi U-3210 (Hitachi Scientific Instruments Ltd., Wokingham, Berks, UK).

Centrifuges – Beckman GPR bench top centrifuge equipped with a GH-3.7 horizontal rotor, Beckman 18 microfuge centrifuge (Beckman Ltd., High Wycombe, Bucks, UK). Kontron T-124 high speed centrifuge with a fixed angle Herolab A8.24 rotor (Kontron Instruments, Watford, Herts, UK).

Microscopy – Zeiss axiophot fluoresence microscope with FITC and rhodamine filters (Carl Zeiss Microscope Division, Oberkochen, Germany), Zeiss axiophot microscope fitted with a computer controlled motorised stage and a triple band pass emission filter (Carl Zeiss Microscope Division, Oberkochen, Germany), Olympus CK2 contrast microscope (Southern Microscopes, Maidst1, Kent, UK).

DNA analysis – Perkin Elmer PCR GeneAmp 2400 (Perkin Elmer, Wellesly, MA, USA), Beckman Coulter CEQ2000XL Sequencer (Beckman Ltd., High Wycombe, Bucks, UK), BioRad 200/2.0 constant voltage power packs (BioRad Lab Ltd., Hemel Hempstead, Herts, UK), UV transilluminator (GRI Ltd., Dunmow, Essex, UK), Horizontal Gel Tank model H5 (Gibco BRL, Gaithersberg, MD, USA).

2.2.2 Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma (Poole, Dorset, UK).

2.2.3 Antibodies

All antibodies were purchased from Cambridge Biosciences (Cambridge, Cambridgeshire, UK) apart from anti-cytochrome c which was purchased from BD Biosciences Ltd. (Cowley, Oxfordshire, UK).

2.2.4 Isotopes

Radioactive isotopes were obtained from ICN Biomedicals (High Wycombe, Bucks, UK)

2.3 Methods Overview

Mitochondria were studied in 12 patients with ALS (sporadic and familial) (table 2.1), 21 controls of similar age (table 2.3), and 10 disease controls (table 2.2). Investigations were performed on muscle tissue, platelets, fibroblasts and myoblasts from each subject, where available. Cell cultures of each tissue type were initiated and grown. These cultures were assayed for respiratory chain enzymes. Platelets were fused with control ρ^0 cells (cells lacking mtDNA) (139), allowing investigation of the platelet mtDNA (124). The same investigations were performed on whole muscle homogenates from patients and controls.

Muscle tissue was analysed histologically, using a number of different stains to look for differences between control and patient samples.

MtDNA analysis was performed on DNA extracted from the muscle tissue. Southern blotting and long-range PCR techniques were employed to assess the total amounts of mtDNA in patients and controls as well as the number of deletions. The SOD1 status of

all patients was analysed by direct sequencing of the PCR-amplified exons and intron-exon boundaries. Modes of inheritance of familial ALS were explored by the analysis of a database of families both with and without SOD1 mutations.

Mitochondrial function of cell and tissue homogenates was assessed by spectrophotometric assay of oxidative phosphorylation enzyme complexes (140-143). Cells for each tissue type were seeded onto glass coverslips, immunostained using antibodies against mtDNA encoded polypeptides, and analysed using a newly developed automated cytometric analysis system. This system allowed a mitochondrial deficit to be quantified in a way that avoids observer bias and can identify even a small proportion of cells not expressing the mtDNA-encoded protein. In addition to looking at the mitochondrial respiratory chain protein complexes, aconitase activity was assayed in whole tissue as a measure of oxidative stress in vivo.

In order to reveal a possible increased sensitivity to oxidative stress and analyse the possible relationship between cell death and oxidative stress, cell cultures were grown in paraquat of varying concentrations and tested for the extent of oxidative stress and apoptosis.

A database of familial ALS patients was created and analysed for evidence of anticipation or sex bias in inheritance of the disease. Given the known inheritance patterns of mitochondrial diseases, trends for unidentified mtDNA mutations were studied.

2.4 Sample Collection

2.4.1 Muscle

With appropriate surgical consent obtained prior to the procedure, open biopsy was performed on the quadriceps muscle, using local anaesthetic, under sterile operating theatre conditions. The sample collected was placed in tissue culture medium and was divided within 2 hours. Half of the piece was frozen at -80°C for whole tissue analysis the other half was used for myoblast generation.

2.4.2 Skin

During the operative procedure for the muscle biopsy, a sliver of skin was taken from the edge of the incision. This was transported to the lab in tissue culture medium and processed for fibroblast generation within 2 hours.

2.4.3 Blood

Blood was collected in a 50 ml syringe using an 18 gauge needle. A tourniquet was applied for the minimum possible time. The blood sample was immediately transferred to a 50 ml container containing 3.8 ml of 10% trisodium citrate, in order to avoid clotting. The sample was transported to the laboratory and processed immediately for platelet collection.

2.5 Cell culture

2.5.1 Overview

Cells were grown on 10-cm culture dishes (Sarstedt). Fibroblasts, ρ^0 and cybrid fusion cells were grown on plain culture dishes using supplemented Dulbecco's Modified Eagle Medium (DMEM) (Sigma) (see appendix 1). Myoblasts were grown on "Cell+" dishes (Sarstedt), in DMEM supplemented with additional foetal calf serum (BioLabs) (see appendix 1). All cell types were grown in an incubator utilising a humidified atmosphere with the CO_2 concentration being maintained at 8% at a constant temperature of 37°C. The growth medium of cell cultures was changed every 3 days.

When cells reached confluence on a culture dish, they were split down. This involved washing the plate with phosphate-buffered saline and adding 1.2 ml of 0.01% trypsin solution (Gibco) in versene (1:5000) (Gibco). The plate was incubated at 37°C (appendix 1) for 1 minute followed by gentle agitation. The cells were then examined under the microscope to ensure that they had dislodged. Once this had taken place, the

trypsin on the plate was neutralised with culture medium. The cells could then either be divided onto other plates to grow further or frozen down.

When freezing cells down, the suspension resulting from trypsinisation was centrifuged at 1300 *g* for 10 min. The supernatant would be discarded. The cells would then be resuspended in culture medium supplemented with 10% DMSO. In this form, the cells would initially be frozen at -80°C overnight in a polystyrene box to ensure slow refrigeration before being transferred to liquid nitrogen for long term storage.

2.5.2 Growing of human myoblasts *in vitro* starting from mononucleated satellite cells

The muscle biopsy specimen was transported to the laboratory in myoblast culture medium. On arrival it was transferred to a sterile 100-ml beaker. Ten ml of myoblast dissociation fluid (see appendix 1) was added. The muscle tissue was gently teased apart for about 3 min using two sterile needles on syringes. The biopsy suspension was transferred to a sterile capped tube and incubated in a water bath or incubator at 37°C for 12 min, whilst shaken slowly (60 rpm).

The capped tube was removed and 10 ml of myoblast culture medium was added. The suspension was swirled gently and triturated by pipetting up and down the muscle fragments through a 10-ml wide bore pipette five times. If this was not possible, then the sterile needles were used again, to break up the fragments.

The muscle fragments were allowed to settle. Using a pipette, the supernatant was drawn off and filtered through a cell strainer (Sarstedt) into a 50-ml tube.

Another 10 ml of dissociation solution was added to the muscle tissue and incubated for a further 12 min.

In the mean time, the supernatant in the 50-ml tube was centrifuged for 10 min to sediment the cells. The supernatant was then aspirated off and the cells were resuspended in 0.5 ml of myoblast culture medium. The tube was subsequently placed in a humidified incubator (37°C and 8% CO_2 in air) with the top slightly loose.

The above process was repeated 2 to 3 times until the muscle tissue was completely dissociated or the 40 ml of dissociation fluid was used up.

The cell pellets resuspended in 0.5 ml of culture medium were combined and made up to 12 ml with myoblast culture medium and transferred to two collagen-coated 6-cm culture dishes (Sarstedt).

The dishes were incubated overnight, humidified, at 37°C and 8% CO_2 in air.

The next day, the medium was aspirated off and the plates rinsed twice with pre-warmed myoblast culture medium to remove red blood cells and cell debris. Fresh medium was then added.

The medium was changed every 3 to 4 days until cells reached a 70% to 80% confluence. Myoblasts were then expanded to 10-cm "Cell+" dishes by trypsinisation. The myoblasts were grown and frozen down as outlined in the cell culture protocols (2.5.1).

2.5.3 Initiating fibroblast cultures

The piece of skin provided from the biopsy was transported to the laboratory in fibroblast cell culture medium. The piece of skin was removed from the transit medium onto a 10-cm culture plate (Sarstedt). Two ml of culture medium was placed onto the sample.

Using a sterile scalpel, the skin sample was divided into approximately 16 pieces on the culture plate. These pieces were equally distributed between two 6-cm culture plates (Sarstedt). A very small amount of medium was added to each plate, so that each piece of skin remained in contact with the base of the dish.

Every 2 days a few drops of medium were added to each plate in order to prevent the samples from drying out. The amount of medium was limited in order that the skin pieces remained in contact with the dish.

Initially, epithelial cells were visible, these were shortly followed by fibroblasts. When the fibroblasts were becoming confluent, they were removed from the plate. This was done by the application of a small amount of trypsin solution (1 ml) (appendix1) and gentle agitation of the plate. The trypsin was neutralized with the addition of 4 ml of fibroblast medium and the cells were transferred to a 10-cm plate.

From this stage, the fibroblasts were grown and frozen down as for the cell culture protocol.

2.5.4 Platelet fusion to ρ^0 cells

Thirty ml of blood was taken from the subject, using an 18-gauge needle, and avoiding the use of a tourniquet. The blood was immediately transferred to a 50-ml tube containing 3.3 ml of 3.8% trisodium citrate. The sample was centrifuged, as quickly as possible, at 200 *g*. The top two thirds of the supernatant was drawn off and transferred to a separate container. The remaining pellet was stored for subsequent DNA extraction. Prostacyclin I (Sigma) was added to this supernatant to a concentration of 70 pM. The sample was centrifuged at 1000 *g* for 30 min.

The supernatant was discarded. The platelet pellet was resuspended in the original volume (supernatant) of Tyrode's buffer (see appendix 1).

Further centrifugation at 1000 *g* for 10 min was performed. This procedure was repeated twice, with the supernatant being aspirated off, resuspended in Tyrode's buffer and centrifuged at the same speed. Following the third spin, the sample was left to stand for 20 min.

A549 ρ^0 cells were grown on 10-cm culture plates using standard fibroblast growth medium (appendix 1). When these were confluent on a culture plate, they were harvested, as outlined (2.5.1), by trypsinisation and centrifugation, and resuspended in 2 ml of Dulbecco's modified Eagle medium without calcium (Sigma).

After 20 min, the platelet suspension was centrifuged at 1000 *g* for 10 min. The supernatant was discarded, and the A549 ρ^0 cells were added to the platelet pellet. This mixture was centrifuged for 5 min at 250 *g*. The supernatant was aspirated off.

Five g of polyethylene glycol 8000 (Sigma) were dissolved in 100 ul of DMSO and 2 ml of calcium free medium, this was added to the cell / platelet pellet and gently agitated for exactly 1 minute. After this minute, 10 ml of medium were added. The suspension was divided between two 10-cm plates and placed in the incubator at 37°C, with a CO_2 concentration of 8%.

The plates were fed regularly, with selective culture (cybrid) medium which lacks uridine and pyruvate and is made up with dialysed foetal calf serum (see appendix 1). This was done to select against cells that had not taken up platelet mtDNA, and hence would be unable to grow without using oxidative metabolism. The cells were grown and harvested according to the cell culture protocol, above (2.5.1).

2.5.5 Mitochondrial Pellet Preparation

A total of 20 confluent plates of the cells being examined were used. The plates were divided into batches of 5. The old media was aspirated off from the first batch, the cells were washed in phosphate buffered saline and trypsinised for 3 min at 37°C. The suspension was then neutralised with fresh media.

The suspended cells from the first batch (5 x 10 ml) were combined in a Falcon tube and spun down at 1300 *g* for 10 min. Whilst spinning, trypsinisation was performed on the second batch.

Upon completion of the spin, the supernatant of batch 1 was discarded. The now pelleted cells were resuspended in the dilute cell suspension from batch 2. This was spun again at 1300 *g* for 10 min.

The process was repeated with successive batches, thereby increasing the concentration of the cells in the suspension each time, until the required number of plates

had been used.

The cells were washed by resuspending the final pellet in 10 ml of PBS, retrieving the cells by centrifugation at 1300 *g* for 10 min. This process was repeated a further two times.

After the third wash, the PBS was discarded and the pelleted cells were frozen (still in the Falcon tube) at -80°C for a minumum of 1 hour.

The second stage of the procedure was performed in the human handling lab in a Class I hood.

The pelleted cells were thawed and resuspended in 2 ml of pre-chilled homogenisation buffer (see appendix 1). All used pipette tips were soaked in vircon for 20 min prior to disposal.

The cells were homogenised in a 2-ml glass homogeniser with a plastic pestle (VWR) for 20 strokes at 1000 rpm.

The homogenised cell suspension was pipetted into a clean (pre-chilled) universal container and spun at 4°C for 10 min at 1500 *g*. The "nuclear fraction" was pelleted as a result of this spin. It contained liberated cell nuclei and membranes in conjunction with (as yet) unbroken cells. The supernatant, known as the post nuclear supernatant contained the cell cytosol, mitochondria and microsomes.

The post nuclear supernatant was carefully removed and stored, on ice, in a clean universal container. The pellet was then resuspended in a fresh 2 ml of pre-chilled homogenisation buffer, the suspension transferred to the homogeniser and the process repeated a further two times in order to harvest the maximum number of mitochondria, combining the post nuclear supernatant from all three spins. The remaining pellet from the third spin was discarded.

The combined post nuclear supernatant was then spun at the same speed once again to pellet any inadvertantly transferred contaminating nuclear fraction.

The post nuclear supernatant was transfered to a fresh (pre-chilled) high speed

Kontron centrifuge tube; the pellet (if any) being discarded.

The post nuclear supernatant was then spun in the Kontron tubes at 4°C for 12 min at 11500 *g*. The post-mitochondrial supernatant was discarded and the crude mitochondrial pellet re-suspended in 500 µl of homogenisation buffer. This was aliquoted into cryotubes (200 µl per tube) and snap frozen in liquid nitrogen. It was stored at -80°C. Enzyme activity was assayed within 5 days.

2.5.6 DNA Extraction from Cultured Cells

The cells required were grown to confluence on two 10 cm cell culture plates. These plates were trypsinised to release the cells, which were collected by centrifugation at 1300 *g* for 10 min. The resulting cell pellet was frozen overnight at -20°C. The procedure for DNA extraction utilised the Nucleon Extraction Kit (Nucleon). One ml of solution B from this kit was added to the defrosted cell pellet and subsequently, the protocol as described below for DNA extraction from blood (2.7.1) was followed.

2.5.7 Confirming Platelet mtDNA Incorporation into Cybrids

An *AluI* (Promega) restriction enzyme digest was performed on DNA extracted from the cultured cybrids in order to assess whether platelet mtDNA had been successfully incorporated. A PCR was performed on the extracted DNA utilising the following reaction conditions;

10 x PCR Buffer (Sigma)	5 µl
DNA	100 ng (1µl)
M60 Primer	50 pmol CTA ACA CCA GCC TAA CCA GA
M61 Primer	50 pmol GGT TAG CAG CGG TGT GTG AG

$MgCl_2$	1.5 mM
dNTP	200 µM
Red Taq (Sigma) (1unit/µl)	2.5 Units
ddH_2O	to 50 µl volume

Reaction Conditions

4 min	94°C	
30 s	94°C	}
30 s	55°C	repeated 30 times
30 s	72°C	}
5 min	72°C	

A restriction enzyme digest was performed using 17.5 µl of these PCR products with *AluI.* Five units of the enzyme, along with acetylated BSA, to a final concentration of 0.1 mg/ml, made up to a volume of 70 µl with the supplied restriction enzyme buffer, were incubated with the products of the PCR reaction for 150 min at 37°C. The resulting digest was run on a high resolution agarose (Sigma) gel of 4% in TAE buffer (appendix 1) with 1 µl ethidium bromide (5.25 mg/ml in H_2O) per 10 ml of gel. The voltage across the electrophoresis tank was set at 25 V for 15 min in order to ensure that the polarity was correct and the marker dye was migrating appropriately. The voltage was subsequently increased to 60 V, and the gel was run for a further 90 min. The gel was examined on an ultraviolet transilluminator. Appropriate Polaroid pictures were taken. The buffer, containing ethidium bromide was poured into an appropriate disposal device. The digest was run with a ϕX174 DNA/*HinfI* marker (Invitrogen).

2.6 Cell Staining

2.6.1 5-Bromo-2'-deoxy-uridine (BrdU) Labelling in Cybrid Cells

The cybrids were grown on glass cover slips to a confluence of around 50%. Staining was performed with a commercially available kit (Roche). Cells were grown for 1 h in fibroblast growth medium supplemented with 15 μM BrdU at 37°C and 8% CO_2.

The cells were removed from the incubator and the labeling medium was removed. The cells were then washed 3 times in beakers containing 25 ml of PBS. The coverslips were drained between washes on tissue paper. Fixation of the coverslips was carried out in 70% ethanol with, 50 mM glycine.HCl (pH 2.0) at -20°C for 30 min. The coverslips were then incubated with 80 μl of anti-Brdu working solution (i.e. anti-Brdu solution (bottle 4 of Roche kit), 10 times diluted in PBS) for 30 min at 37°C in a humidified atmosphere. Having been washed three times in PBS, the cover slips were ncubated with secondary antibody (anti-mouse-Ig-Alexa 488) (Molecular Probes) at a dilution of 1:100 in PBS for 30 min at 37°C in a humidified atmosphere.

The cover slips were washed again 3 times in PBS, before being mounted on glass slides (Agar) with Citifluor (Agar). Examination of the cover slips was perofrmed using a Zeiss axiophot fluoresence microscope equipped with FITC and rhodamine filters. The poportion of cells which demonstrated nuclear staining without extra-nuclear staining were compared with the proportion of cells which demonstrated extra-nuclear staining.

2.6.2 Mitochondrial protein immunofluorescence staining of cultured cells

The cultured cells to be examined were seeded onto coverslips for 24 h in appropriate growth medium (appendix 1). This was replaced with prewarmed medium containing 4 μM (50 μg per 35 ml of medium) Mitotracker® Red CM-H2XRos (Molecular Probes, Inc.) for 45 min. A stock of 50 μg Mitotracker dye in 200μl of DMSO was made up beforehand. The cells were then cultured for 30 min in plain prewarmed medium.

The coverslips were washed three times in beakers containing 25 ml of PBS (not supplemented with $MgCl_2$ and $CaCl_2$). The coverslips were drained between washes on tissue paper.

The coverslips were fixed in fresh, pre-warmed, 4% paraformaldehyde in PBS for 20 min. The coverslips were washed three times in beakers containing 25 ml of PBS. Between washes, the coverslips were drained on tissue paper. The coverslips were placed in a rack in a container with PBS.

The rack was then transferred with coverslips to a container containing methanol at -20°C for 15 min. The coverslips on the rack were then placed in a container containing PBS.

The coverslips were further washed three times in beakers containing 25 ml of PBS, draining the coverslips between washes on tissue paper. Blockage of non-specific protein binding sites on the coverslips was performed with 300 µl of 10% normal goat serum (Sigma) in PBS for 30 min at 37°C in a humidified atmosphere.

The coverslips were drained and incubated with 80 µl of anti-COX1 antibody (Molecular Probes) at a dilution of 15 µg IgG/ml in PBS, 2% normal goat serum for 45 min at 37°C in a humidified atmosphere.

Coverslips were washed a further three times in beakers containing approximately 25 ml of PBS. Between washes the coverslips were drained on tissue paper.

The coverslips were incubated with 80 µl of 100 × diluted goat anti-mouse IgG Alexa® 488 (Molecular Probes, Inc.) in PBS, 2% normal goat serum for 45 min at 37°C in a humidified atmosphere.

The coverslips were washed 3 times in beakers containing approximately 25 ml of PBS, draining the slips between washes on tissue paper. The coverslips were placed in a rack in a container containing PBS.

The coverslips were mounted on glass slides in Citifluor/PBS/glycerol (Agar) supplemented with 1 µg/ml of DAPI (Sigma) and sealed with nail polish.

The slides were stored at 4°C in the dark until examination under fluorescence microscope could take place.

2.7 Superoxide Dismutase 1 Mutation Analysis

2.7.1 DNA Extraction from Whole Blood - Using Nucleon BACC2 kit (Amersham Biosciences)

The blood from which the DNA was to be extracted was placed in a 50-ml centrifuge tube. Four volumes of reagent A from the Nucleon BACC2 kit were added to the blood. The tubes were agitated for 4 min at room temperature. The tubes were spun at 1300 *g* for 5 min. The supernatant was removed from each tube. Two ml of reagent B from the Nucleon BACC2 kit was added to each pellet. The pellets were vortexed briefly in order to disperse them within the reagent. The suspension was transferred to a clean 15-ml tube. Fifteen µl of RNAase were added to each tube, and they were incubated at 37°C for 30 min.

Five hundred µl of 5 M sodium percholate was added to each tube, and they were inverted twelve times. Two ml of chloroform was added to each tube and they were inverted. Without further mixing, 300 µl of separation-suspension was added to each tube. The tubes were spun at 1300 *g* for 3 min.

The upper phase from each tube was removed and transferred to a clean 15-ml tube. Two volumes of cold absolute ethanol was added to the upper phase and the tubes were inverted twelve times. The DNA precipitated out, allowing its removal. The DNA was carefully transferred to a 1.5-ml Eppendorf tube, containing 1 ml of 70% ethanol.

The Eppendorf tubes were spun at maximum speed (11500 *g*) for 5 min. The ethanol was poured off, and the Eppendorf tubes were left on a drying block for evaporation of the remaining ethanol. One ml of TE buffer (appendix1) was added to each tube, and the tubes were left overnight for the DNA pellet to dissolve.

2.7.2 Spectrophotometric Assay of DNA Solutions

The Hitachi U-3220 spectrophotometer was set up in order to measure the absorption between wavelengths of 210 nm and 310 nm. Scanning speed was set at 120 nm per minute. A baseline was obtained by measuring the absorption of 480 µl of distilled water in a 500 µl quartz cuvette (Sigma). Twenty µl of the DNA sample, obtained by the extraction protocol was added to the cuvette and mixed thoroughly. Absorption was then measured. To determine the concentration from this reading in g/l, the absorption at 260 nm was divided by 20 (extinction co-efficient for DNA) and multiplied by 25 (volume in cuvette / volume of DNA added),

2.7.3 PCR for SOD1 Exon 1

Primers : G107 – 5' CCC CTT GGC CCG CCC CAG TCA TTC 3'

G108 – 5' TCG GGA GCG GCC TCG CAA CAC AAG 3'

Master Mix (for 25 µl); (all in µl)

H_2O	11.65
10 X Buffer (Sigma)	2.5
50 mM $MgCl_2$	0.75
dNTP (200 mM)	0.5
G107 (50 pM)	2.0
G108 (50 pM)	2.0
Red Taq (Sigma) (1unit/µl)	2.0
DMSO	2.6
DNA (1 µg/µl)	1.0

Reaction Conditions :

94°C	5 min	
94°C	30 s	}
52°C	30 s	repeated 30 times

72°C	30 s	}
72°C	7 min	

2.7.4 PCR for SOD1 Exon 2

Primers : G109 – 5' AAG TGG TCA GCC TGG GAT TTC 3'

G110 – 5' AGG GGC TAC TCT ACT GTT TAT 3'

Master Mix (for 25 µl); (all in µl)

H_2O	11.65
10 X Buffer	2.5
50 mM $MgCl_2$	0.75
dNTP (200 mM)	0.5
G109 (50 pM)	2.0
G110 (50 pM)	2.0
Red Taq (1unit/µl)	2.0
DMSO	2.6
DNA (1 µg/µl)	1.0

Reaction Conditions :

94°C	5 min	
94°C	30 s	}
51°C	30 s	repeated 30 times
72°C	30 s	}
72°C	7 min	

2.7.5 PCR for SOD1 Exon 3

Primers : G105 – 5' TTG TTT CTG TTC CCT TCT CAC TGT 3'

G106 – 5' GTT CCC CTT TGG CAC TTG TAT T 3'

Master Mix (for 25 µl); (all in µl)

H_2O	14.25
10 X Buffer	2.5
50mM $MgCl_2$	0.75
dNTP (200mM)	0.5
G105 (50 pM)	2.0
G106 (50 pM)	2.0
Red Taq (1 unit/µl)	2.0
DNA (1 µg/µl)	1.0

Reaction Conditions :

94°C	4 min	
94°C	30 s	}
52°C	30 s	repeated 30 times
72°C	30 s	}
72°C	7 min	

2.7.6 PCR for SOD1 Exon 4

Primers : G97 – 5' TGT TTG CAA ATT TGT GTC TAC TCA 3'

G98 – 5' GAA TTC GCG ACT AAC AAT CAA AGT 3'

Master Mix (for 25 µl); (all in µl)

H_2O	16.6
10 X Buffer	2.5
50 mM $MgCl_2$	1.5
dNTP (200 mM)	0.5
G97 (50 pM)	0.3
G98 (50 pM)	0.6

Red Taq (1unit/µl)	2.0
DNA (1 µg/µl)	1.0

Reaction Conditions;

94°C	5 min	
94°C	30 s	}
50°C	30 s	} repeated 30 times
72°C	30 s	}
72°C	7 min	

2.7.7 PCR for SOD1 Exon 5

Primers : G103 – 5' TTT CTG CTT TTA AAC TAC TAA 3'

G104 – 5' TTT AAG TCT GGC AAA ATA CAG 3'

Master Mix (for 25 µl); (all in µl)

H_2O	13.65
10 X Buffer	2.5
50 mM $MgCl_2$	0.75
dNTP (200 mM)	0.5
G103 (50 pM)	0.8
G104 (50 pM)	1.2
Red Taq (1unit/µl)	2.0
DMSO	2.6
DNA (1 µg/µl)	1.0

Reaction Conditions;

94°C	5 min	
94°C	30 s	}

48°C	30 s	repeated 30 times
74°C	30 s	}
74°C	7 min	

2.7.8 Agarose Gel Electrophoresis of SOD1 PCR Products

A 100ml Agarose gel was made up at 1.5%, using TAE buffer (see appendix 1). The gel was left to cool until around 60°C. Ethidium bromide (5 µl of 10 mg/ml) was added to the gel, and it was poured into a horizontal plastic gel tank containing a comb and left to set.

TAE buffer was poured into the gel tank, covering the surface of the gel. The plastic comb was removed and the gel loaded with 5 µl of PCR product mixed well with 1 µl of loading dye (Promega). One well was loaded with 3 µl of 100 bp DNA standard ladder (Promega) which had been mixed with 1 µl of loading dye.

The voltage across the electrophoresis tank was set at 25 V for 15 min in order to ensure that the polarity was correct and the marker dye was migrating appropriately. The voltage was subsequently increased to 60 V, and the gel was run for a further 90 min. The gel was examined on an ultraviolet transillmuinator. Appropriate Polaroid pictures were taken. The buffer, containing ethidium bromide was poured into an appropriate disposal device.

2.7.9 DNA Sequencing

The PCR product was cleaned using the Qiaquick Qiagen© clean-up kit. The DNA concentration was then determined by spectrographic analysis using the DNA spectrophotometric protocol, as outlined above (2.7.2). The sample was sequenced in both directions, using the primers that were used in the initial PCR process. Samples were sent for sequencing to the Wolfson Institute, University College London. A Beckman Coulter CEQ 8000 genetic analysis systems was used. The raw data was analysed using

the *Seqman* programme and compared with available SOD1 sequence data (human chromosome 21 sequence segment 31953890 – 31963107 with accession number NC_000021).

2.8 Biochemical Assay of Mitochondrial Respiratory Chain Complexes

2.8.1 Spectrophotometry Overview

Assays to determine mitochondrial respiratory chain activity were performed using spectrophotometric methods. Separate assays were performed for complex I (NADH-ubiquinone Q reductase), complex II/III (succinate cytochrome c reductase) and complex IV (cytochrome c oxidase) activity (140-143). The samples were assayed for citrate synthase activity, and the final enzyme activity was expressed as a ratio of activity per citrate synthase activity. In addition, tissue samples were also analysed for protein, so that this could be controlled for.

The assays were performed blind. After preparation from cell pellets, the mitochondrial aliquots were assigned numbers by a third party, with the assignation of the individual aliquots not being revealed until after the assays and activity calculations had been performed. Assays for each complex were performed on three separate samples, with an average taken of the results to be used in calculating the enzyme activity, as described below. For the purposes of standardization, two patient samples and two age- and sex-matched controls were grown, harvested and analysed at the same time. All the samples were assayed using the same reagents on the same spectrophotometer (Hitachi).

2.8.2 Complex I Activity

This assay was performed using ubiquinone (Q_1) (Eisai Corporation. Tokyo, Japan). A working solution of 1 in 50 dilution was made from a stock sample. The required amount of working solution for the assay was calculated by loading two quartz cuvettes with 990 µl of ethanol and adding 10 µl of the ubiquinone working solution.

Both cuvettes were placed in the spectrophotometer, set to measure absorbance at 275 nm wavelength. A granule of sodium borohydride was added to the reference (back) cuvette, in order to reduce the ubiquinone to a non-absorbing ubiquinol state. The final absorbance change was measured and used to calculate the amount of ubiquinone required (see the spectrophotometry calculation section, below).

The mitochondrial enzymes were exposed by freeze-thawing the mitochondrial preparations (which were prepared as described above) three times in liquid nitrogen and a water bath set at 30 °C.

The spectrophotometer was set to perform a time scan at a wavelength of 340 nm at a temperature maintained at 30°C, with sampling set to a frequency interval of 10 s and total scan time at 8 min.

Three reference and three test cuvettes were set up with 800 µl of 25 mM phosphate buffer (pH 7.2) (containing 10 mM $MgCl_2$), 30 µl of 5 mM NADH, 10 µl of 100 mM potassium cyanide and 50 µl of bovine serum albumin. One hundred µl of distilled water was added to the reference cuvettes. The test cuvettes were given 100 µl of distilled water minus the volume of ubiquinone (amount derived as above) and the volume of the mitochondrial preparation to be used. Mitochondrial preparation was then added to the test cuvettes. Both sets were loaded into the spectrophotometer (the test cuvette at the back, the reference cuvette at the front) which was auto-zeroed. The assay was then started and left for around 30 s for the absorbance to stabilise. The calculated volume of ubiquinone was added to the test cuvette, and the assay left to run for 2 to 3 min. At this point 10 µl of rotenone (10 µM stock solution in ethanol) was added to both cuvettes. The rotenone-sensitive cellular NADH dehydrogenase activity was, therefore, inhibited. The rate was followed for a further 2 to 3 min, and the rotenone-sensitive rate of reaction was then calculated as described below. Three assays were performed for each sample.

2.8.3 Complex II/III activity

The mitochondrial enzymes were exposed by freeze-thawing the mitochondrial preparations (which were prepared as described above) 3 times in liquid nitrogen and a water bath set at 30°C.

The spectrophotometer was set to perform a time scan at a wavelength of 550 nm at a temperature maintained at 30°C, with sampling set to a frequency interval of 10 s and total scan time of 8 min.

Six cuvettes (three test and three reference) were loaded with 600 µl of 166 mM phosphate buffer (pH 7.4), 20 µl of 15 mM EDTA and 50 µl of 2 mM horse cytochrome c (Roche). The three reference cuvettes were loaded with 320 µl of distilled water. Test cuvettes were loaded with 320 µl less the volume of mitochondrial preparation to be used plus 40 µl (for the volume of succinate required). Three Eppendorf tubes were loaded with 10 µl of 100 mM potassium cyanide and 40 µl of 500 mM sodium succinate. In order to activate the mitochondrial enzyme complex, the previously freeze-thawed samples were aliquoted at the required amount into the Eppendorf tubes 5 min before the start of the assay. At 1 min prior to the start of the assay, 10 µl of 100 mM potassium cyanide was added to the reference cuvette. The assay was started and auto-zeroed. After the baseline had stabilized, the contents of the pre-warmed Eppendorf tube was added to the test cuvette and mixed thoroughly. After spectrophotometric measurement running for 3 to 4 min, 10 µl of 500 mM antimycin A were added to both cuvettes. The trace was then checked for an absence of antimycin A – insensitive activity.

For each subsequent assay, the mitochondrial preparations were added to the Eppendorf tubes in a water bath set at 30°C at 5 min prior to the start of the assay, while the potassium cyanide was not added to the reference cuvette until 1 min prior to the start of the assay. The rate of reaction, and enzyme activities were calculated as described below. Three assays were performed for each sample.

2.8.4 Complex IV (cytochrome c oxidase) activity

The mitochondrial enzymes were exposed by freeze-thawing the mitochondrial preparations (which were prepared as described above) three times in liquid nitrogen and a water bath set at 30°C.

The spectrophotometer was set to perform a time scan at a wavelength of 550 nm at a temperature maintained at 30°C, with sampling set to a frequency 10 s and total scan time of 8 min.

Initially the volume of stock reduced horse cytochrome c was calculated. The method of reduction is described below (2.8.5). Two cuvettes were identically loaded with 100 µl of 0.1 M phosphate buffer (pH 7.0) and 850 µl of distilled water. Fifty µl of the reduced cytochrome c was added to both cuvettes. Ten µl of 0.1 M potassium ferricyanide were added to the back (reference) cuvette. The change in absorbance was recorded and used to calculate the volume of reduced cytochrome c (50 µM) required for the assay, using the calculation described below.

Three reference and three test cuvettes were loaded with the previously calculated amount of reduced cytochrome c and 900 µl of 0.1 M phosphate buffer (pH 7.0), less the volume of cytochrome c to be used and the volume of mitochondrial preparation (for the test cuvettes), or the 10 µl (of ferricyanide) for the reference cuvettes. The reference cuvettes were loaded into the back of the spectrophotometer, while the test cuvettes were loaded into the front. Autozeroing was carried out, before 10 µl of ferricyanide was added to the reference cuvette in order to completely oxidise the cytochrome c. At this point, the absorbance read around 0.96 and the assay was started. The extinction coefficient for reduced cytochrome c is 19.2 x 10^3 M^{-1} 550 nM. Thus, an absorbance of 0.96 implies a reduced cytochrome concentration of 50 µM.

After 30 s, the mitochondrial preparation was added, as rapidly as possible, to the test cuvette without removing the cuvette from the spectrophotometer. The assay was then left to run for 5 min. Data were taken from time point zero (at the addition of the mitochondrial preparation), and then again at 1 min intervals. The rate of reaction

constant (K) was calculated using a logarithmic scale (see paragraph 2.8.7.6). This figure was then used to calculate the enzyme activity as described below.

2.8.5 Reduction of Cytochrome c

Cytochrome c was reduced in order to carry out the complex IV assays, using ascorbate and subsequent dialysis.

Five ml of ascorbate, at a concentration of 12 mg in 100 ml was added to 100 ml of horse cytochrome c (Roche) at a concentration of 10 mg/ml and was mixed thoroughly for an hour. The sample was then placed in a dialysis membrane and dialysed against 0.01 M phosphate buffer (pH 7.0) at 4°C for 2 h. After this time, the buffer was changed, and dialysis continued for a further hour.

In order to check that the cytochrome c was fully reduced, two cuvettes were loaded with 850 µl of distilled water, 100 µl of phosphate buffer (pH 7.0) and 50 µl of the cytochrome c solution. They were loaded into the spectrophotometer, with wavelength measurement set to 550 nm. Ten µl of the ascorbate solution (50 ml/ml) was then added to the back cuvette. No change in absorbance indicated that full reduction had taken place.

In order to ensure that there was no ascorbate in the reduced cytochrome c samples, two cuvettes were loaded in a similar way to that described above. Instead of ferricyanide, oxidized cytochrome c was added to the back cuvette. The principle was that the absorbance would change if this was reduced by any residual ascorbate. On the basis of these analyses, a decision was made regarding the need for further dialysis, further ascorbate, or preparedness for use in the assay, proper. If ascorbate was present in the sample, further dialysis was performed. If the sample was shown to have undergone oxidation, further ascorbate was added, and the dialysis repeated.

2.8.6 Citrate Synthase Activity

Citrate synthase is an enzyme of the mitochondrial matrix, and, as such, may be used to establish the concentration of mitochondria within a sample. The enzyme rates that were derived from the assay were expressed as ratios to the same sample's citrate synthase activity.

The spectrophotometer was set to perform a time scan at a wavelength of 412 nm at a temperature maintained at 30°C, with sampling set to a frequency interval of 10 s and total scan time at 8 min. Because the citrate synthase is not a membrane-bound complex, Triton-X100 was used to solubilise the membranes and release the enzyme.

One reference cuvette and three test cuvettes (per sample) were loaded with 500 µl of 50mM Tris buffer (pH 8.0) (see appendix 1), 20 µl of 10 mM acetyl CoA, 20 µl of 10 mM DTNB and 10% (volume) of Triton-X100. Four hundred and forty µl of distilled water was added to the reference cuvette. Four hundred and forty µl of distilled water less the volume of mitochondrial preparation to be used and 10 µl (for the oxaloacetic acid to be added) were added to the test cuvette. Both cuvettes were loaded into the spectrophotometer (test at the front and reference at the back). The required volume of mitochondrial preparation was added to the test cuvette and the assay started. When the baseline had stabilized, 10 µl of 10 mM oxaloacetic acid were added to the test cuvette. The assay was allowed to run for a further 5 min. Enzyme activity was calculated as described below.

2.8.7 Spectrophotometry Calculations

2.8.7.1 Calculation of volume of working solution of ubiquinone

A 1 M solution of ubiquinone will produce an absorbance change at 275 nM of 12.25×10^3. Therefore, if volume V of ubiquinone produces absorbance change A, the molarity of the stock solution is $A/12.25$ (in mM). The required molar concentration

within the cuvette is 50 μM. Therefore, 50×10^{-3} / ((absorbance change/12.25)/*V*) is the new volume of ubiquinone stock that needs to be added to the assay.

2.8.7.2 Complex I (NADH-Ubiquinone1 reductase):

The complex I activity was calculated by dividing the change in absorbance at 440 nM over time before and after the addition of rotenone to the reaction (fig 2.2) to determine the rate of reaction. This rotenone sensitive rate can be used to calculate enzyme activity, by knowing that the molar extinction coefficient of NADH is 6.81×10^3 M. The rotenone sensitive rate of NADH reduction is:

$$\{[(A_B-A_A) / (T_B-T_A)-(A_E-A_D)/(T_E-T_D)] / 6.81 \times 10^3\} \times 1000/v \times 1/1000$$

Where A is the absorption at a given time point, T is the time at a given point and v is the volume of the mitochondrial sample added to the cuvette (in μl). The rate is given in $mol.min^{-1}.ml^{-1}$.

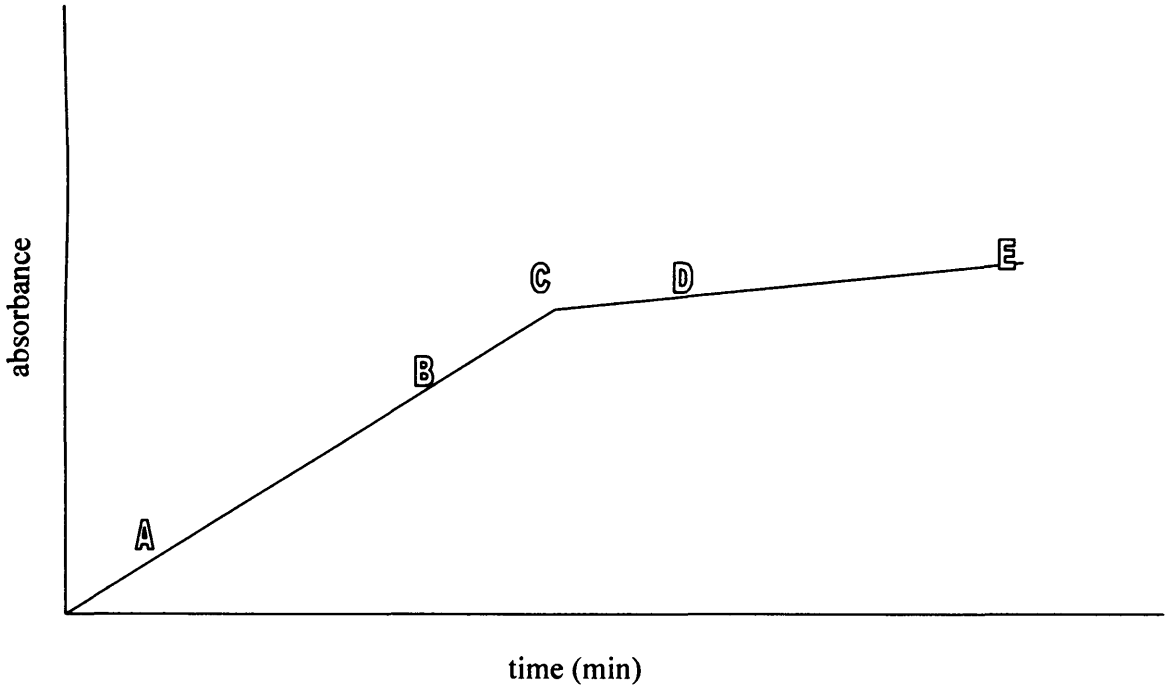


Figure 2.2 The rate of reaction of mitochondrial respiratory chain complex 1 Where C represents the point of addition of rotenone.

2.8.7.3 Complex II (succinate cytochrome c reductase)

Follows the basic principle of the above calculation, but the molar extinction coefficient of cytochrome c at 550 nm is $19.2 \times 10^3 M^{-1}.cm^{-1}$. There is one rate to use (fig 2.3) and the formula changes:

$$\{[(A_B-A_A) / (T_B-T_A)] / 19.2 \times 10^3\} \times 1000/v \times 1/1000$$

Where A is the absorption at a given time point, T is the time at a given point and v is the volume of the mitochondrial sample added to the cuvette (in µl). The rate is given in $mol.min^{-1}.ml^{-1}$.

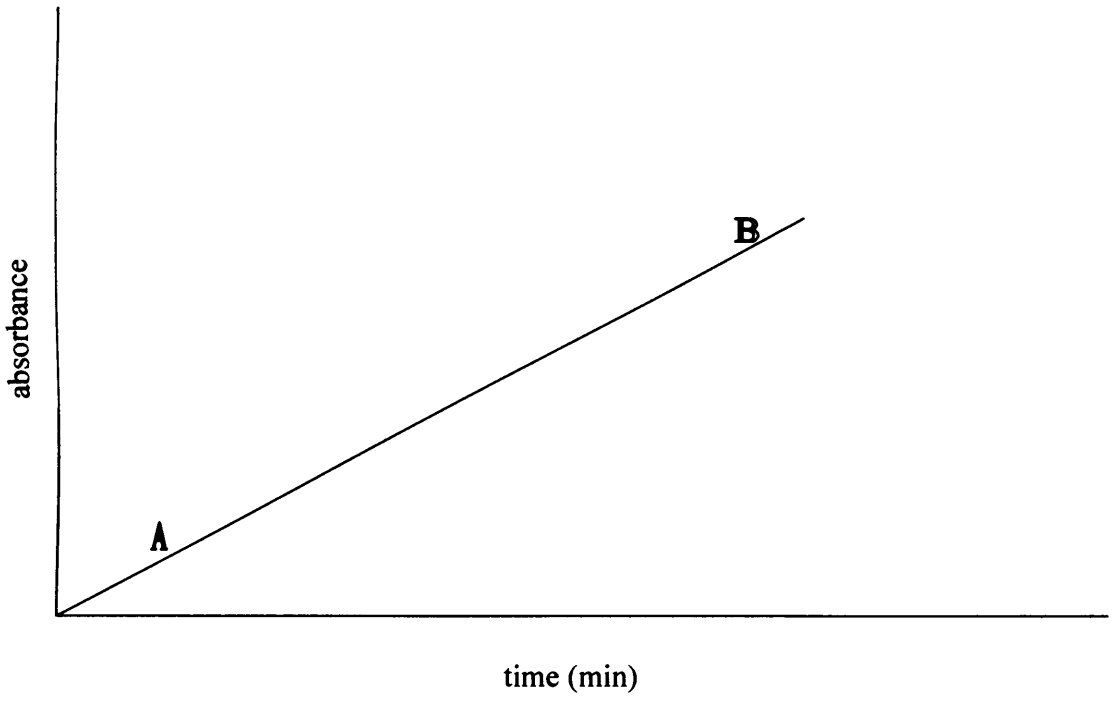


Figure 2.3 The rate of reaction of mitochondrial respiratory chain complex II/III

2.8.7.4 Citrate Synthase

The equation is for above, but the extinction coefficient of DTNB at 412nm is $13.6 \times 10^3 M^{-1}.cm^{-1}$

2.8.7.5 Volume of reduced cytochrome c to use in complex IV assay.

Spectrophotometry was performed as above. The difference in absorbance between the cuvettes was recorded. As the molar extinction coefficient for cytochrome c at 550 nm is 19.2×10^3, it can be calculated that 50 μM of reduced cytochrome c will produce an absorbance change of 0.96. Because the amount of reduced cytochrome c in each batch varies, it is appropriate to calculate the volume required for each batch that is used. As 50 μM of reduced cytochrome c is required for the assay, the volume of reduced cytochrome c to be added to the cuvette: $(0.96/A) \times V$ (where A is the observed absorbance and V is the volume of reduced cytochrome c used).

2.8.7.6 Complex IV (cytochrome c oxidase) activity.

The rate of reaction is first-order (fig 2.4), therefore, the pseudo-first rate constant, *K,* was calculated (13g). This was done by noting the absorbance values at 1 minute intervals following the initial reading (at the point of addition of the mitochondrial preparation). A plot was then made of $\ln A_{t=0} - \ln (A_{t=0} - A_{t=n})$ against time:

$$K = \ln A_{t=0} - \ln (A_{t=0} - A_{t=n}) \text{ per minute}$$

The results were expressed as K. $min^{-1}.mg^{-1}$ or K. min^{-1}.(citrate synthase activity)$^{-1}$. Taking into account the dilution of the sample in the cuvette and the protein concentration or citrate synthase activity of the sample.

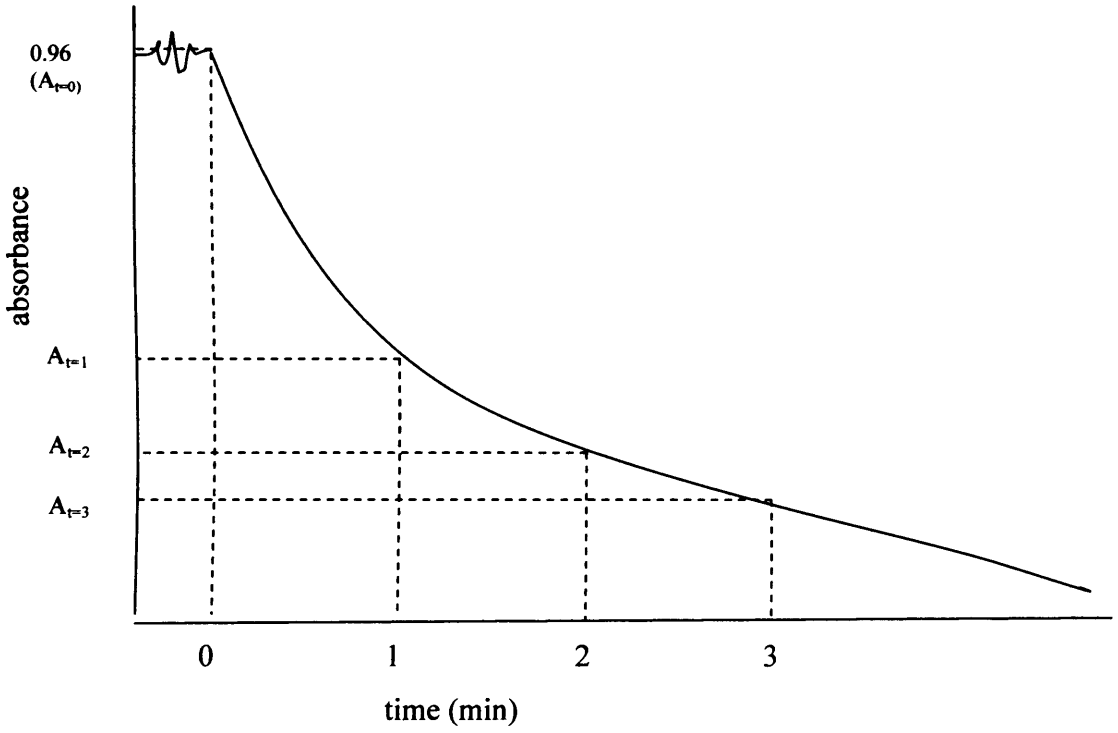


Figure 2.4 The rate of change in absorbance of cytochrome c in the calculation of the activity of the mitochondrial respiratory chain enzyme IV

2.9 Muscle Analysis

In addition to the biochemical assays carried out on myoblasts, fibroblasts and cybrid cells, whole muscle taken from the open biopsy was homogenised and analysed for the respiratory chain enzymes and aconitase, a marker of oxidative damage. Respiratory chain enzyme assays were performed on muscle homogenate as described for cell mitochondrial preparations (2.6).

2.9.1 Muscle Homogenisation

Following biopsy, a piece of muscle tissue, stored at -80°C was taken from the freezer and snap frozen in a 2-ml cryotube in liquid nitrogen. This was done because

fragmentation of the muscle was more complete from a solid state. The sample was then placed in a homogenisation tube with 75 µl of homogenisation buffer (appendix 1). Using the homogenisation pestle at 200 rpm, the sample was homogenised for 20 complete strokes. In order to clarify the sample, the muscle homogenate was spun at 450 *g* for 1 min. The resulting supernatant was distributed between two 2-ml cryo-tubes and placed on ice for subsequent respiratory complex assays, performed as described for cell homogenates (2.8).

2.9.2 Aconitase Assay

The mitochondrial enzymes in the sample were exposed by freeze-thawing three times in liquid nitrogen. The spectrophotometer was set to record at a wavelength of 340 nm with a run time of 35 min. Sampling was set at a frequency interval of 10 s. Three test and one reference cuvettes were loaded with 600 µl of 83.3 mM tris buffer (pH 7.4), 100 µl of 4 mM NADP, 50 µl of 100 mM sodium citrate, 10 µl of 60 mM $MgCl_2$, 10 µl of Triton X-100 and 2 units of citrate dehydrogenase. The required amount of homogenate was added to the test cuvettes, and the volume of each cuvette was made up to 1 ml with distilled water. The cuvettes were left in a water bath for 30 min at 30°C for the enzyme to become activated. The samples were loaded into the spectrophotometer and the assay run for 30 min. The reaction rate was calculated as for the respiratory chain enzymes (2.8.7), with a molar extinction coefficient of NADPH of $6.2 \times 10^3.M^{-1}.cm^{-1}$ being used.

2.9.3 Protein Determination

As well as being corrected for citrate synthase activity, the muscle homogenate enzyme activities were also corrected for protein concentration. This was done using a commercially available kit (Bio-Rad). The principle of this technique is that coomassie blue dye binds to protein under acidic conditions and a shift in light absorbance from 465 nm to 565 nm occurs. The same technique for protein estimation was used for the cell aconitase assays.

One ml of the standard coomassie dye in phosphoric acid and methanol solution (Bio-rad Laboratories) was diluted with 4 ml of water. On a 96-well plate (flat bottomed), 200 µl of this solution were plated into 8 standard wells and an appropriate number of sample wells (4 per sample being analysed). A standard of bovine serum albumin at 2 mg/ml was diluted 1:10 to give a working solution of 200 µg per ml. Standard concentrations of 0, 3, 6, 9, 12, 15, 18, 21 and 24 µg/ml were obtained by removing the required amount of dye solution from the standard wells and adding the same amount of protein solution. In a similar way, the sample was added to the sample wells at at least two different concentrations. The samples were mixed within their wells, and the plate was placed onto an ELISA plate reader (Hitachi), set to measure at 600 nm. The standard solutions gave a curve of absorbance against concentration, from which the protein concentration of the sample wells could be calculated from their absorbance.

2.9.4 Light Microscopy

A portion of the muscle sample obtained at biopsy was divided in theatre by the pathology technician (Ms J Workman), and snap frozen in isopentane cooled with liquid nitrogen. The sample was transported to the laboratory where the same technician performed standard lactate dehydrogenase, haematoxylin and eosin, succinate dehydrogenase, cytochrome oxidase, Gomori trichome stains for fibre size, type and distribution on each sample. The slides were then placed into storage. The slides were viewed using a 40× objective lens on a Zeiss light microscope. Any abnormal features present on each slide were noted. Comparison was made between the muscle biopsy samples taken from the patient group, controls and disease controls to determine whether there were features more common or exclusive to the patient group. The slides were also viewed and reported by Professor AHV Schapira; these reports were compared with the data obtained by the author in order to confirm that any abnormal features present had been correctly identified.

Electron microscopy was performed by Dr R King on the sample from the patient with familial disease, and reported verbally. The muscle biopsies were all prepared and

stained using standard protocols in the Department of Clinical Neurosciences, Royal Free and University College Hospital Medical School.

2.10 MtDNA analysis

2.10.1 DNA extraction from whole muscle

The muscle tissue (approximately 100 mg) was macerated with two scalpels on a microscope slide in the Class 1 hood. The tissue was transfered to a 1.5-ml Eppendorf tube containing:

600 µl of 0.1 M sodium EDTA, 0.01 M Tris•HCl (pH 8.0)

40 µl of 10% SDS

100 µl of 20 mg proteinase K per ml.

The mixture was digested for 3 h at 56°C, rotating in an incubator. When the material was fully digested, the tubes were stored at -20°C until further processing could take place. Subsequently, the sample was thawed and 750 µl of liquefied phenol, washed in Tris buffer (Fisher Scientific) were added in a Class 1 hood. Extraction with phenol took place by rotation at room temperature for 15 min. The mixture was then micro-centrifuged at 1.14×10^4 *g* for 15 min (room temperature), and the (upper) aqueous phase was transfered very carefully to a new 1.5-ml Eppendorf tube. Extraction was then performed with 700 µl of phenol-solution/bromo-chloropropane/isoamyl alcohol (25:24:1) by rotation at room temperature for 15 min. The mixture was micro-centrifuged at 1.14×10^4 *g* for 10 min (room temperature), transferring the aqueous phase carefully to a new 1.5ml Eppendorf tube. This extraction step was repeated a further three times. Extraction with 650 µl of bromo-chloropropane/isoamyl alcohol (24:1) was then performed by rotation at room temperature for 15 min. After micro-centrifuging the sample at 1.14×10^4 *g* for 5 min (room temperature), 500 µl of the aqueous phase was transferred to a new 1.5-ml Eppendorf tube. Fifty µl of 3 M NaOAC (pH 4.8) and 1.1 ml of ethanol (room temperature) were added and mixed thoroughly. The DNA precipitate

was collected by centrifugation at 1.14×10^4 *g* for 10 min (room temperature), the supernatant was removed and 1 ml of 70% ethanol was added to the tube. The pellet was collected by centrifugation at 1.14×10^4 *g* for 10 min (4°C), and the supernatant removed. Having been briefly (5 min) dried at 37°C in the heat block, the pellet was dissolved in 30-100 µl of TE buffer (appendix 1) (i.e at room temperature for several hours). Once the DNA was fully dissolved, the quantity and quality were assessed with spectrophotometric assay (2.7.2).

2.10.2 Restriction Enzyme Digest

The DNA samples obtained as described were pipetted into 8-µg aliquots. Three µl of *PvuII* restriction enzyme (Promega) and 6 µl of 10x restriction enzyme buffer were added to each sample. The aliquots were made up to 60 µl, mixed throughly and briefly spun at 1.14×10^4 *g* in a centrifuge before being incubated at 37°C for 2 hours.

2.10.3 Southern Blotting

The genomic samples obtained from the restriction digest were mixed with 0.2 volumes of loading dye and run, along with a *Hin*dIII-digested λ DNA marker on a 0.7% agarose gel in 1 × TAE (appendix 1), 0.5 µg/ml ethidium bromide using the Pharmacia GNA 200 gel tank. The samples were resolved at 135 V on the gel, until the bromophenol blue band marker was running off the end of the gel. The gel was then transferred to a shallow container and depurinated in 330 ml of 0.2 M HCl for 10 min whilst being agitated.

The depurinating solution was carefully poured off and the DNA was denatured in 330 ml of 0.5 M NaOH, 1.5 M NaCl, for 30 min whilst being agitated. The denaturing solution was changed after 15 min. The denaturing solution was poured off and the gel neutralised in 330ml of 1 M Tris·HCl (pH 7.4) and 1.5 M NaCl for 40 min whilst being agitated. The neutralising solution was changed after 20 min.

During neutralisation of the gel, the Optiblot Blotting Unit (Scotlab) was prepared for blotting. The unit, including the wick, was soaked for 10 min in an excess of single distilled water. The water was changed once during this period. Finally, the unit was rinsed with 10 × SSC buffer (appendix 1). Six hundred ml of 10 × SSC was poured into the base of blotting unit. A wet sheet of 3 MM paper (Whatmann) (in 10 × SSC) was transferred to the middle of the wick of the blotting unit. The gel was rinsed briefly in 10 × SSC and transferred onto the wetted 3 MM filter paper. A Hybond-N membrane (Amersham Biosciences) was soaked, first in ddH_2O, then in 10 × SSC, and placed on top of the gel. Three 3MM filter papers were soaked in 10 × SSC and placed in succession on to the top of the membrane.

The edges of the gel-membrane were covered with long strips of parafilm to prevent leakage. Fifteen absorbant dry towels were placed onto the gel-membrane and, finally, the top of the blotting unit. After 18 hours, the blotting unit was disassembled. The Southern blot DNA was cross-linked with UV light by placing on a UV-transilluminator (GRI) for exactly 2.5 min. The blot was dried between a thick paper towel and stored at room temperature until use.

Prehybridisation of the blot was carried out in 10 ml of 6 × SSC, 5 × Denhardt's solution (appendix 1), 0.5 % SDS, 10 μg/ml heat-denatured, sonicated salmon sperm DNA (Sigma) at 65°C in an incubator (Hybaid) for 1 h. Because of local safety guidelines, all work involving radioactive materials was performed by Dr Taanman. ^{32}P-labelled heat-denatured 18S RNA gene DNA and/or mtDNA probes, prepared by Dr Taanman using a commercially available labelling kit (Amersham) were added to the tube and left to hybridise overnight at 65°C. The blot was washed in 50 ml of pre-warmed 2 × SSC in the tube at 65°C for 15 min. The blot was then washed in 400 ml of pre-warmed 2 × SSC in a large container in a water bath at 65°C for 15 min. The blot was then washed in 250 ml of pre-warmed 2 × SSC, 0.1% SDS in the large container at 65°C for 15 min. The blot was washed once more in 250ml of pre-warmed 2 × SSC, 0.1% SDS in the large container at 65°C for 15 min. Finally the blot was washed in 500 ml of prewarmed 0.1 × SSC, in a large container at 65°C for exactly 10 min.

The amount of radioactivity present was assessed with a Geiger counter. All radioactive products and waste were disposed of in accordance with local protocols and regulations.

The blot was initially exposed in a PhosphorImager cassette (Amersham) and the levels of bound probe were quantified using ImageQuant software (Amersham). Student's t-test was peformed on the results in order to detect a difference in the mean quantities of DNA.

2.10.4 Long-range PCR

DNA, extracted from skeletal muscle (see 2.10.1) was used for long-range PCR analysis of mtDNA. This technique utilised the Expand Long Template PCR System (Roche, Mannheim, Germany). A forward primer corresponding to the mtDNA nucleotide positions 6222-6236 and a reverse primer corresponding to the mtDNA nucleotide positions 16133-16153 were used (5'-CCC TCT CTC TCG CAT and 5'-CAG GTG GTC AAG TAT TTA TGG).

In order to determine the optimal concentration of primers and DNA template to use with the kit, a number of trial PCRs of control and patient DNA were performed using differing amounts of reagent.

The optimum amounts of reagent were determined to be (for 50 µl reaction):

DNA (1 µg/µl)	0.2 µl
dNTP (100 nM/µl)	2 µl
Forward primer (100 nM/µl)	3 µl
Reverse primer (100 nM/µl)	3 µl
10 x buffer	5 µl
Enzyme mix	0.75 µl
H_2O-	36.05 µl

The PCR was carried out with a 1 min initial denaturation period at 93°C followed by 10 cycles of 10 s of denaturation at 93°C, 30 s annealing at 55°C and 6 min elongation at 68°C. This was followed by 20 cycles of 15 s denaturation at 93°C, 30 s annealing at 55°C and elongation at 68°C, with each elongation period being 20 s longer than the one that preceded it in the cycle. Final elongation was carried out for 7 min at 68°C.

The samples were then run on a 0.8% agarose gel in 1 x TAE buffer for 5 hours at 100 V, and visualised on a UV transilluminator.

2.11 Cellular Response to Oxidative Stress

2.11.1 Cell Apoptosis Assays

Capase-3 is a protease which is activated during the process of programmed cell death (apoptosis) (144). Apoptosis was measured in cell cultures. A commercially available kit (PhiPhiLux™ (Calbiochem)) was used for the assay of intracellular caspase-3 activity in response to a model of oxidative stress. The assay is based on the reaction of the enzyme with the cell-permeable PhiPhiLux® G_1D_2 substrate. Each substrate molecule contains two fluorophores and the cleaved substrate fluoresces green.

Cells of the experimental cultures at an equivalent passage number were grown on 16-mm glass cover slips to a similar cell density. Cells were analysed at baseline and following 24 hour incubation in growth medium supplemented with a stock solution of 1mM paraquat (25.7 g/l) to make up 0.1 μM (0.1 ml paraquat stock in 1000 ml cell growth medium) and 1 μM (1 ml paraquat stock in 1000 ml cell growth medium) solutions. The cover slips were washed once in PBS and then 40 μl of 10 μM of substrate peptide from the kit was added to each cover slip. The coverslips were incubated at 37°C for 30 mins and washed again with PBS. Without delay, the coverslips were examined under a fluorescence microscope using a fluorescein filter at 40 x 10 magnification. The number of fluorescent cells in one field was counted. The lamp of the microscope was then turned on, allowing a count to be made of all the cells in the field using phase contrast. Three randomly selected fields, progressing from left to right across the cover

slip to avoid counting any cells twice, were analysed in this way. Caspase activation was recorded as the number of fluorescent cells per total cell population. For each culture performed this process was carried out on three different cover slips, with an average being taken of the total number of fields analysed.

2.11.2 Oxidative damage assay

As already described (2.9), aconitase was utilised as a measure of oxidative damage. In order to assess the level of oxidative damage in response to oxidative stress, aconitase levels were measured in cell cultures exposed to 0.05 μM 0.1 μM or 1 μM paraquat solutions in growth medium for 24 hours. The cell suspensions were harvested by trypsinisation of two confluent 10-cm plates of the cells followed by centrifugation at 1300 *g* for 10 min and resuspension of the pellet in 70 μl of PBS. Twenty μl of suspension of cells were used for each assay. The method of assay is decribed in the section above (2.9.2). Values for aconitase activity were corrected for protein concentration of the cell suspension being analysed.

2.12 Data Analysis

All data were entered and stored in Microsoft Excel and Word Programmes. Subsequent statistical analysis of the data was carried out using SPSS© (version 12.0) software application. Individual statistical tests that were used are indicated in the methodology for each experiment, where appropriate. Statistical significance was taken as a calculated p value of less than 0.05 in all cases.

3 Analysis of Nuclear DNA from Controls and Patients with ALS to Determine the Presence of Superoxide Dismutase 1 Mutations

3.1 Aims

To analyse the nDNA of the ALS patients, normal controls and disease controls to determine the presence or absence of mutations of the Superoxide Dismutase 1 (SOD1) gene.

3.2 Introduction

Sporadic and familial forms of ALS exist. The pattern of inheritance is usually dominant with variable penetrance (67). Mutations of the SOD1 gene, which encodes the enzyme copper/zinc superoxide dismutase, are found in approximately 20% of familial patients and occasionally, apparently sporadic, patients (67). SOD1 mutations account for 1-2% of all patients with ALS (ALS1) (48). Other, rarer, genetic abnormalities have been detected in patients with ALS, including the alsin (ALS2) (53) and senataxin (ALS4) (60) genes, along with linkage data for undefined genes (ALS3 (59), ALS5 (61), ALS6 (145), ALS7 (62) and ALS8 (63)) but the significance of these is uncertain at the present time (table 1.1). All 12 of the patients in this study (table 2.1) were screened for mutations of the SOD1 gene. One of the patients (PA11) was known to have familial ALS.

3.3 Approach

In order to screen for SOD1 mutations, total genomic DNA was extracted from whole blood (2.7.1), and the concentration determined by spectrophotometric methods (2.7.2). This DNA was subjected to PCR for the 5 exons of the SOD1 gene using the primers, reagents and conditions detailed (2.7.3). The samples were sent for sequencing

at the Wolfson Institute for Biomedical Research, UCL. The sequence data was analysed using DNA STAR software package.

3.4 Results

There were no mutations in any of the SALS patients, disease controls or normal controls for the 5 exons of SOD1. The familial ALS patient was found to have a heterozygous 1147 T→C mutation in exon 4. This mutation is predicted to result in a Ile113Thr amino acid substitution in the protein (Figure 3.1).

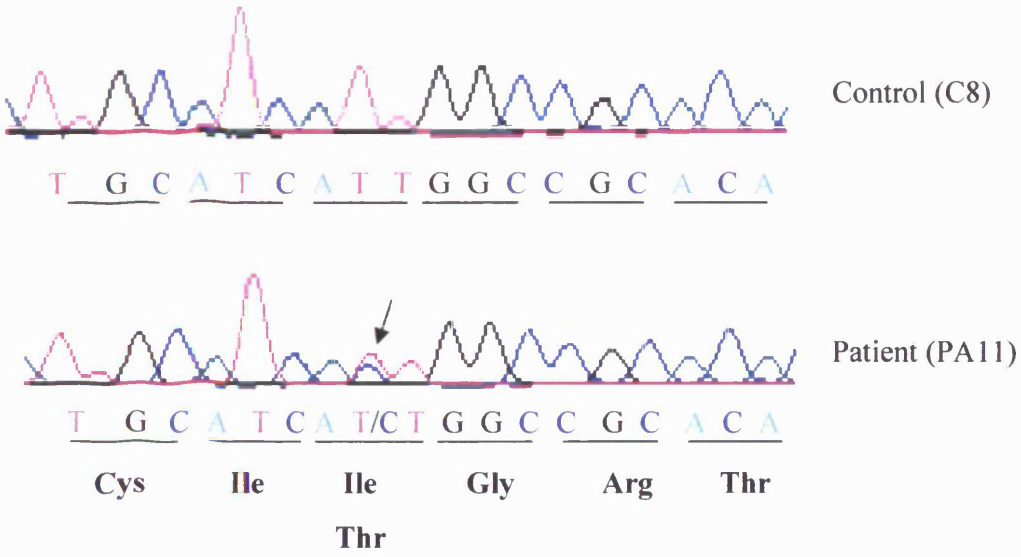


Figure 3.1 Heterozygous 1147 T→C mutation in SOD1 exon 4 (arrowed) producing a Ile/Thr substitution.

3.5 Discussion

As none of the sporadic ALS patients in this study had a family history of neurological disease, it is not surprising that there were no SOD1 mutations detected in this cohort. Although mutations in SOD1 of apparently sporadic cases are recognised (51)

these are relatively rare. The familial patient studied showed no distinctive results in any of the investigations carried out into mitochondrial function.

The mutation identified (1147 T>C) on SOD1 exon 4 is a well recognised mutation in FALS and was first reported in 1993 (47). The patient's mother died of ALS in her fifties, and genetic studies were never performed on her. None of the patient's siblings has developed ALS as yet.

4 Histological Features of Skeletal Muscle in ALS

4.1 Aims

To examine muscle tissue taken from patients with ALS, comparing it with normal control and disease control muscle to determine whether there are any histological features specific to ALS, and what these abnormalities may be.

4.2 Introduction

ALS is primarily a disorder of the motor neurons. As a part of the diagnostic workup, patients may have muscle biopsy with light microscopy of the samples obtained (146). This can be especially important where there is some doubt about the diagnosis or where there are a number of atypical features of ALS present (147). Some authors have reported abnormal metabolic activity (148) and ultrastructural abnormalities of the mitochondria in the skeletal muscle of patients with ALS (149). Skeletal muscle fasciculation and wasting are prominent clinical features of ALS. In view of the evidence, as outlined above, that there may be abnormal mitochondrial function and ultrastructure of skeletal muscle in ALS, features of mitochondrial diseases, as well as more general denervating conditions were looked for in the skeletal muscle samples which were obtained for biopsy.

Muscle biopsies are generally performed for diagnostic purposes, where the histological appearance of the muscle may provide additional information toward the management of the patient. Different histochemical stains are used to look for different pathological features. Haematoxylin and eosin (H+E) stains are used to look at fibre architecture and morphology (figures 4.1 – 4.4). Localisation of the nuclei and invasion of inflammatory cells are also seen with this stain. The presence of mitochondrial pathology may be looked for with cytochrome oxidase (for COX negative) or succinate dehydrogenase stains as well as Gomori trichome stains looking for the presence of

ragged-red fibres which are indicative of mitochondrial accumulations. The absence of activity of these enzymes due to mitochondrial abnormalities can be seen in individual fibres of the muscle tissue (figure 4.8). ATPase stains, performed at different pH levels differentiate between type I and type II muscle fibres. These fibres perform different functions in the skeletal muscle. Type 1 fibres are slow contracting and predominantly aerobic in their metabolism whereas type II fibres are short acting, predominantly anaerobic in their metabolism. Different disease states can influence the distribution and relative densities of the two fibre types. Sudan black stains lipid present in the muscle whilst acid phosphatase demonstrates the presence of macrophages. There are a number of different conditions for which muscle histology analysis is important. Investigation of the muscular dystrophies, inflammatory disorders, storage disorders and mitochondrial diseases may involve the processing and analysis of a muscle biopsy.

4.3 Approach

Skeletal muscle biopsies of 12 patients with clinically definite ALS were examined and compared with skeletal muscle samples from a number of other conditions. Muscle biopsy specimens from 9 individuals with denervating conditions and benign fasciculation syndromes (table 2.2) were analysed along with muscle biopsy samples from 10 normal individuals. The same range of stains was carried out for each sample. ATPase, acid phosphatase, cytochrome oxidase, haematoxylin and eosin, Sudan black and also Gomori trichrome stains were performed according to standard laboratory protocols by Mrs Jane Workman. The samples were analysed, for each stain performed, in blinded fashion, and all of the positive features which were identified were confirmed as being present on another independent histological assessment.

The positive features identified and quantified for each sample were :

Variation in fibre size (figure 4.1)

Internal nuclei (figure 4.2)

Fibre atrophy (figure 4.3)

Vacuolation (figure 4.4)

Grouping of all fibres (figure 4.5)

Grouping of type 1 fibres (figure 4.6)

Grouping of type 2 fibres (figure 4.7)

Lysosomal activity

COX negative fibres (figure 4.8)

Ragged-Red fibres (figure 4.9)

Inflammatory infiltrate (figure 4.10)

Increased lipid content (figure 4.11)

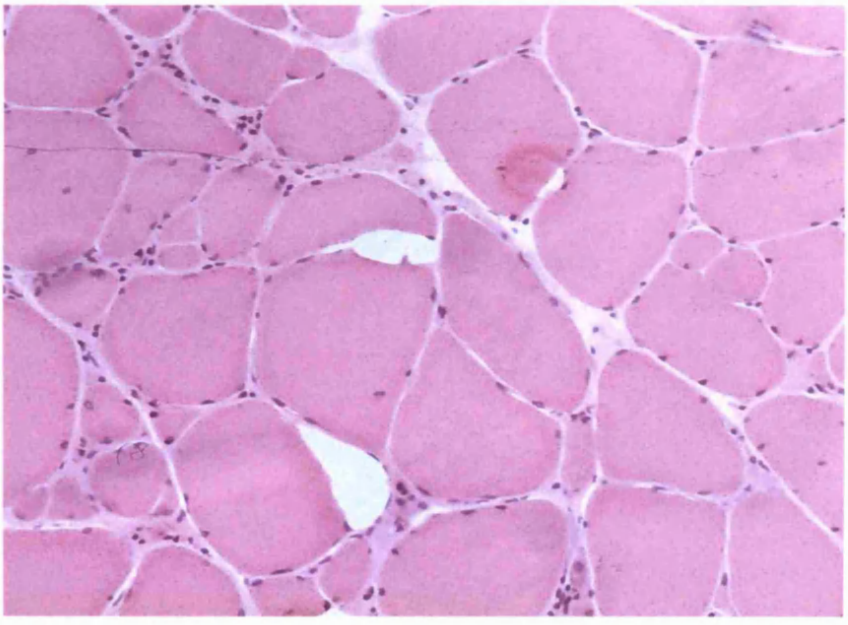


Figure 4.1 – Variation in fibre size for sample C7 on H and E staining

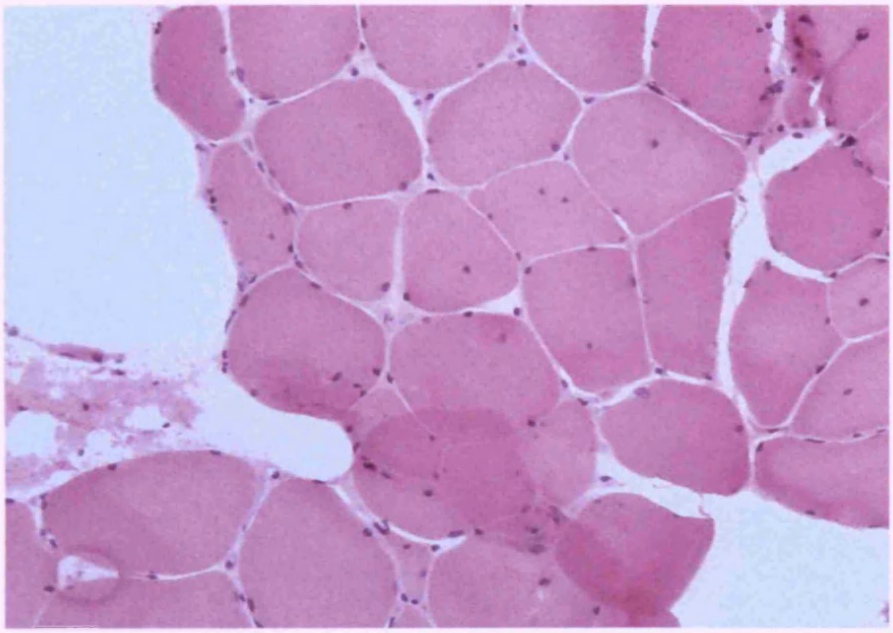


Figure 4.2- Numerous internal nuclei seen for sample C7 on H and E staining

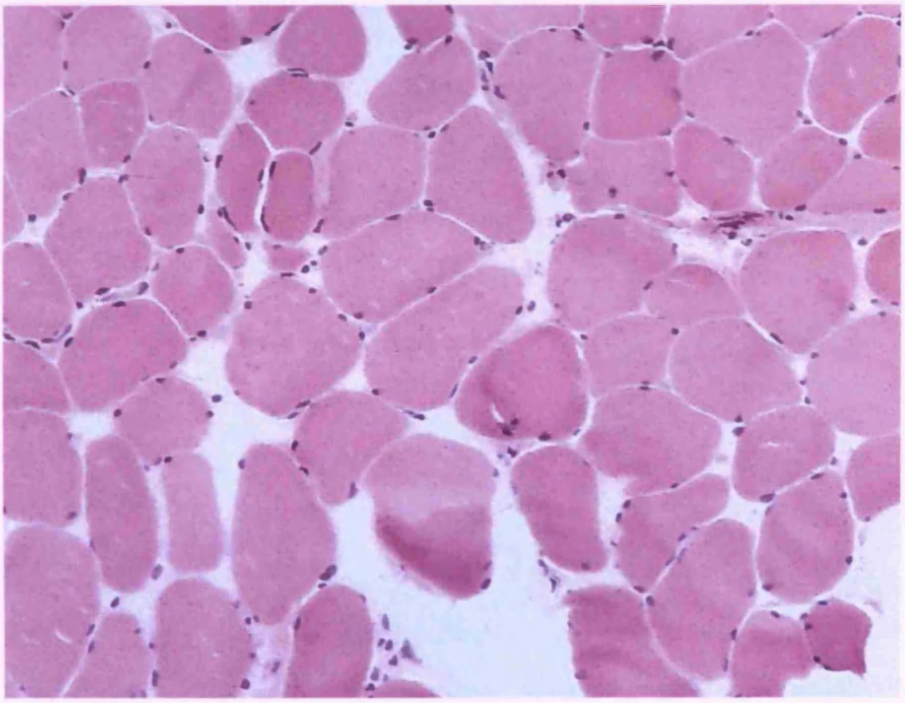


Figure 4.3 – Fibre atrophy seen on sample C11 with H and E staining

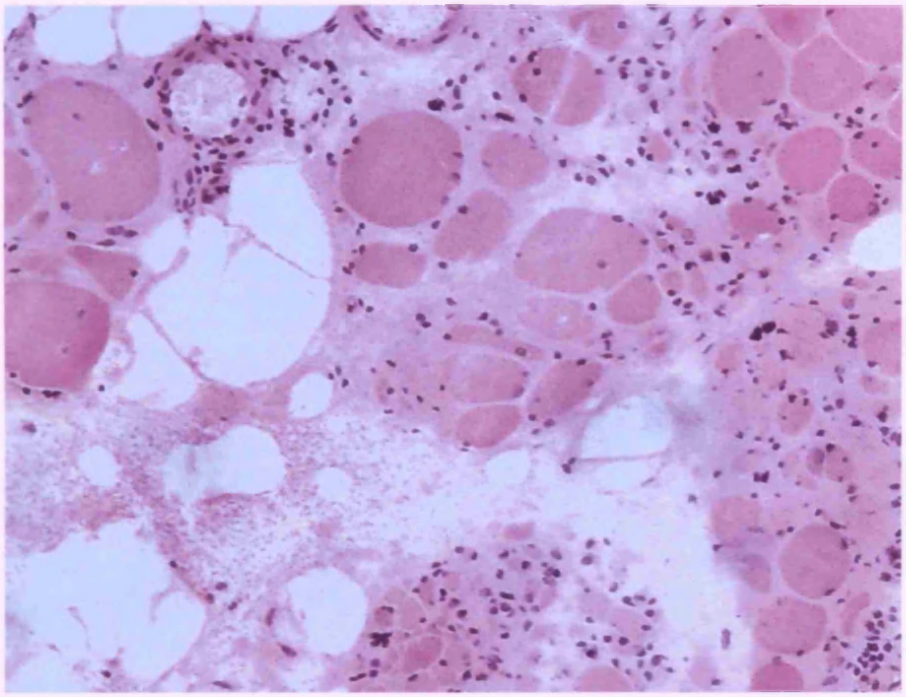


Figure 4.4 – Vacuolation seen on sample C11 with H and E staining

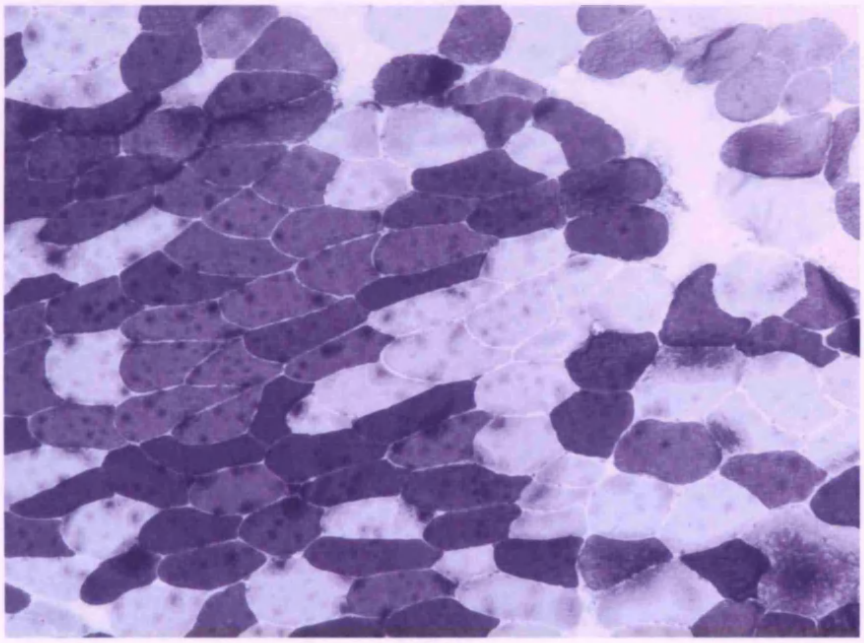


Figure 4.5 – Grouping of type I (light) and type II (dark) fibres seen on sample PA2 with ATPase staining at pH 4.6

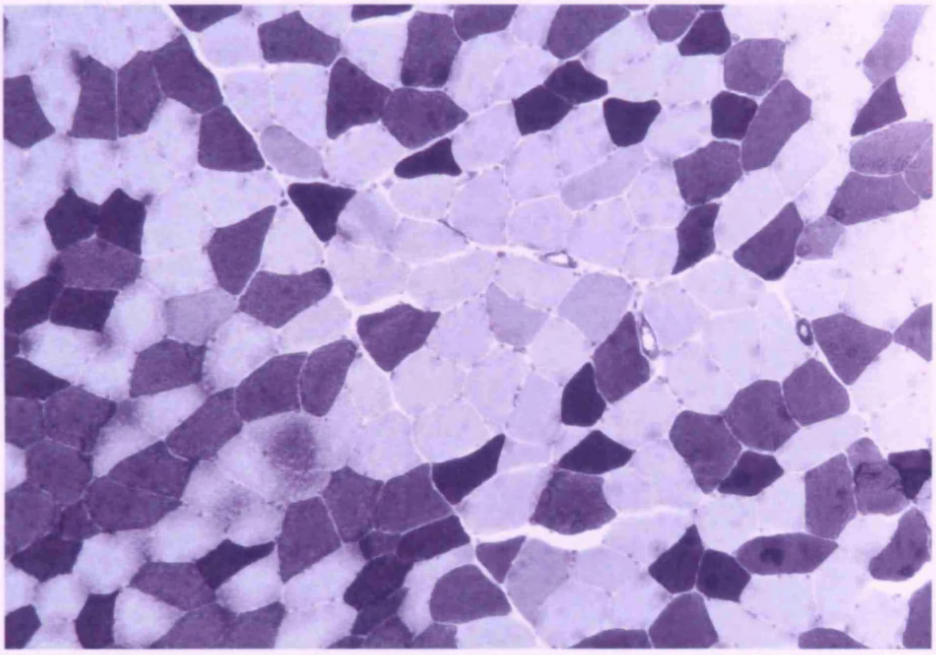


Figure 4.6 – Grouping of type I (light) fibres seen for sample D3 with ATPase staining at pH 4.6

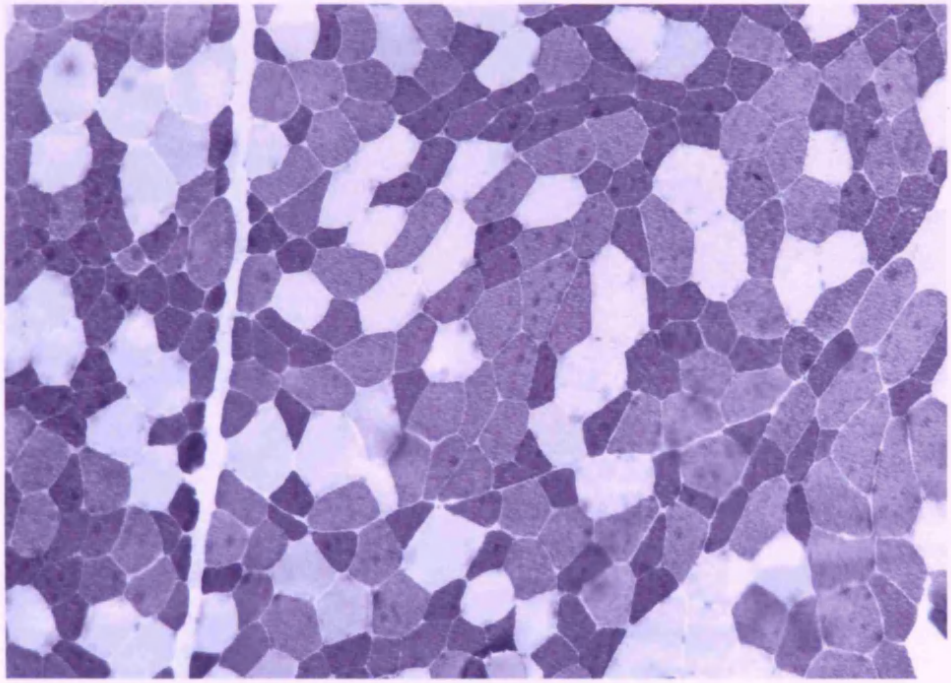


Figure 4.7 – Grouping of type II (dark) fibres from sample D1 with ATPase staining at pH 4.6

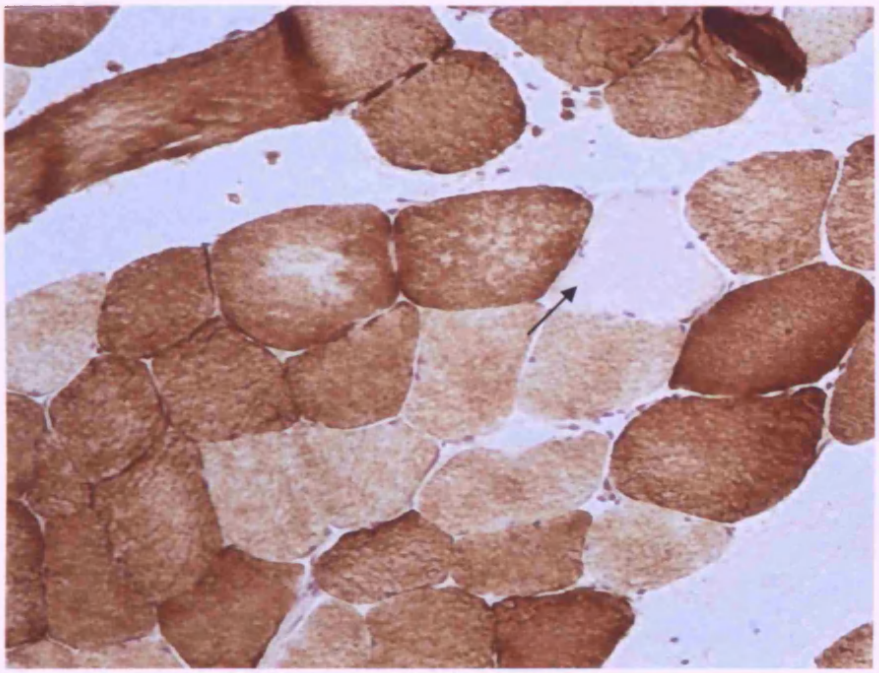


Figure 4.8 – COX negative fibre (arrowed) on sample PA4 with cytochrome oxidase staining

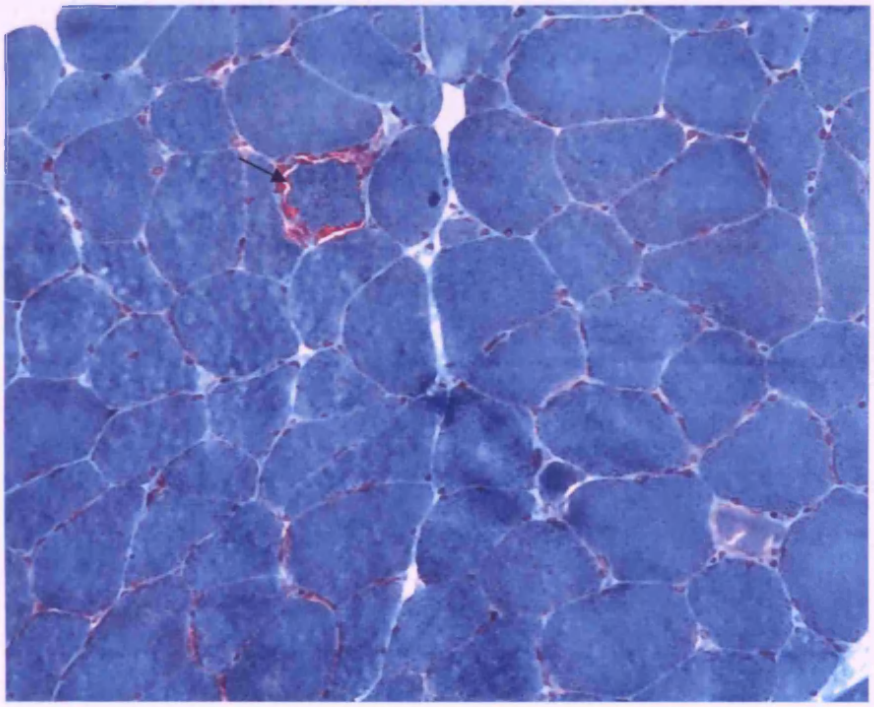


Figure 4.9 – Red-ragged fibre (arrowed) seen on sample D5 with Gomori trichrome staining

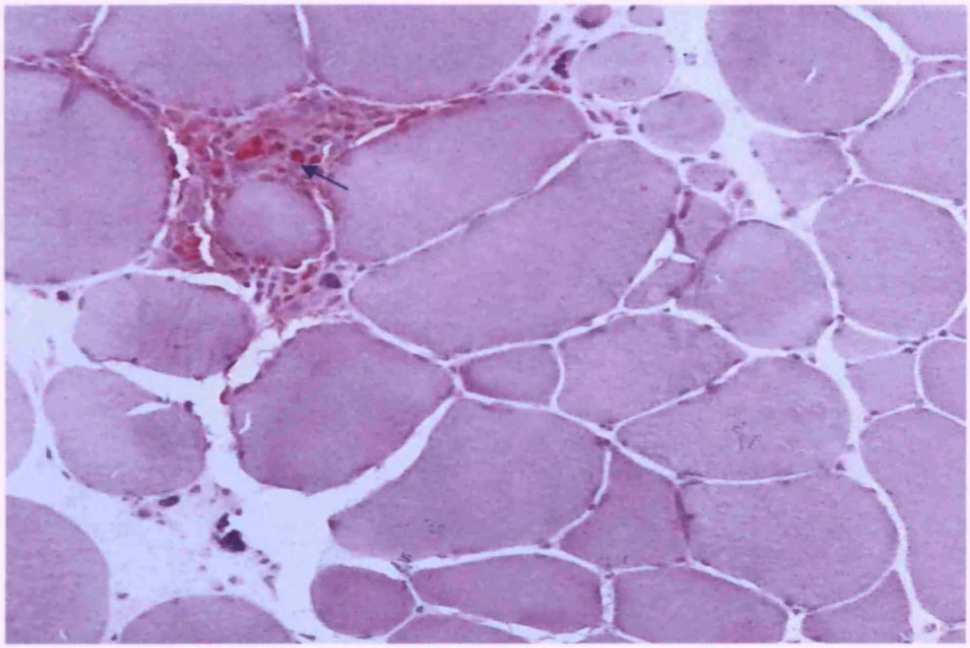


Figure 4.10 – Inflammatory infiltrate (arrowed) seen on sample PA1 using acid phosphatase staining.

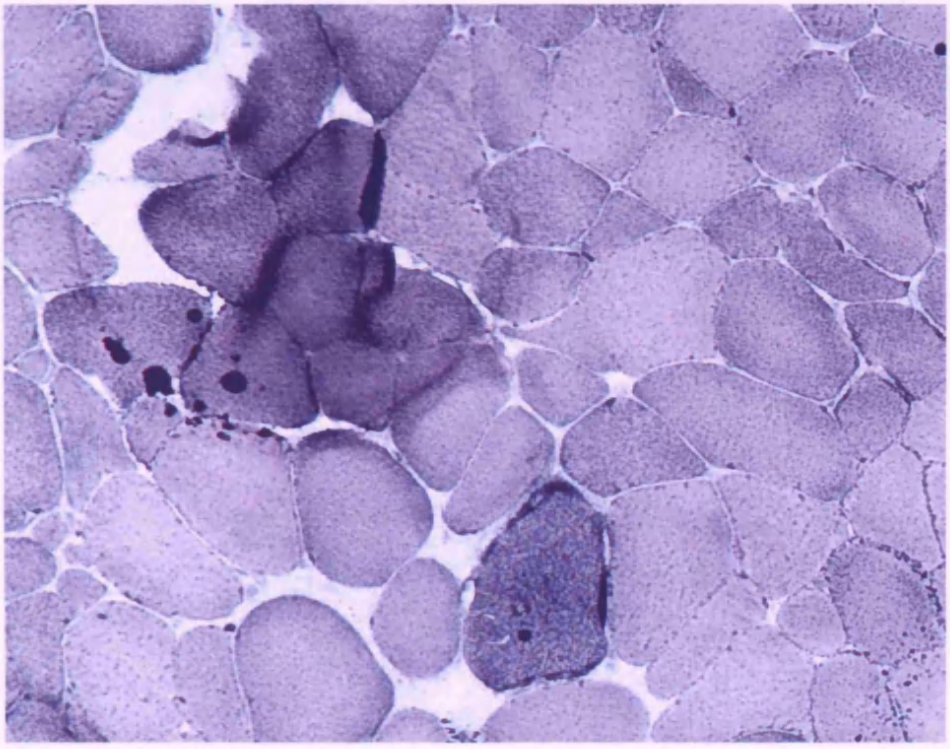


Figure 4.11 – Increased lipid content in sample C9 seen with Sudan black staining

4.4 Results

The samples from patients with ALS demonstrated a number of different abnormalities associated with denervation, such as fibre atrophy, angulation and variation in size. Statistical analysis using a three-way χ^2 test revealed that these were not distinctive or diagnostic (figure 4.12 / table 4.1). Grouping of all fibre types, occurred with a greater frequency amongst patient muscle samples than in control or disease control samples, although this did not quite reach significance (p=0.063).

The muscle biopsy specimen from the FALS patient was subject to electron microscopy by Dr R King. This was reported as demonstrating no significant abnormalities. There were no distinguishing features on immunocytochemical analysis.

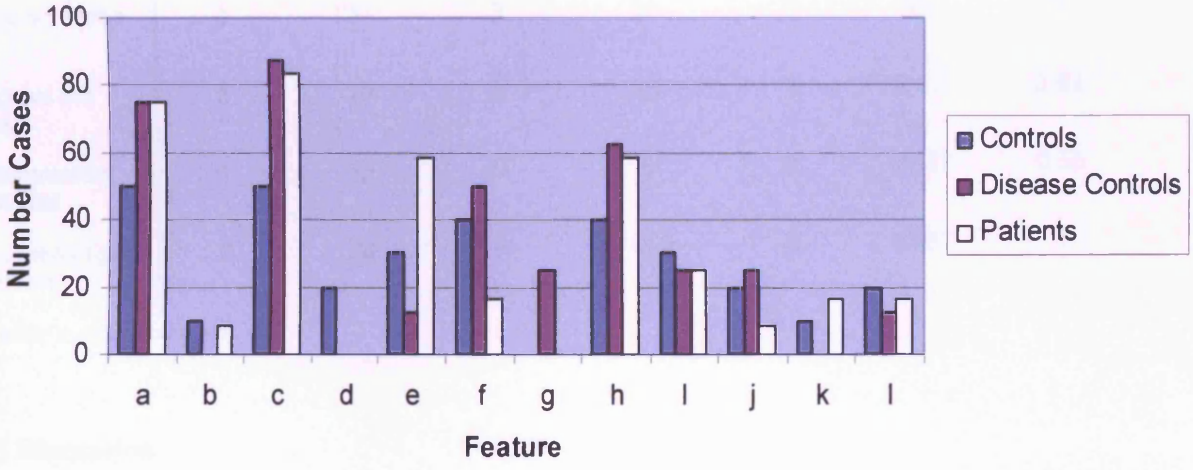


Figure 4.12 Bar chart showing the rates of occurrence of each histological feature in the control, disease control and patient groups. a = variation in fibre size, b = internal nuclei, c = fibre atrophy / angulation, d = vacuoles, e= grouping of all fibre types, f = grouping of type 1 fibres, g = grouping of type 2 fibres, h = lysosomal activity, i = COX negative fibres, j = ragged red fibres, k = inflammatory infiltrate, l = increased lipid content

Table 4.1 – Features seen in diagnostic muscle biopsy as % of total samples with statistical analysis.

Feature	Controls (No)	Controls (%)	Disease Controls (No)	Disease Controls (%)	Patients (No)	Patients (%)	3-way Chi squared* (p value)
Variation in fibre size	5	50	6	75	9	75	0.69
Internal nuclei	1	10	0	0	1	8.33	1
Fibre atrophy / angulation	5	50	7	87.5	10	83.33	0.42
Vacuoles	2	20	0	0	0	0	0.5
Grouping of all fibres	3	30	1	12.5	7	58.33	0.063
Grouping of type 1 fibres	4	40	4	50	2	16.67	0.24
Grouping of type 2 fibres	0	0	2	25	0	0	0.5
Lysosomal activity	4	40	5	62.5	7	58.33	0.72
COX -ve fibres	3	30	2	25	3	25	1
Ragged red fibres	2	20	2	25	1	8.33	0.62
Inflammatory infiltrate	1	10	0	0	2	16.67	0.55
Increased lipid content	2	20	1	12.5	2	16.67	1

**Fisher's exact test used*

4.5 Discussion

While muscle biopsy is important in excluding other conditions, where these are suspected, there are no histopathological abnormalities which, alone, would be diagnostic of ALS. Although grouping of all fibre types occurs with a greater frequency in muscle from patients with ALS, this feature is not specific enough to be regarded as having diagnostic value *per se*, as it is more a function of a denervation-reinervation pattern as recognised in previous studies (150,151). The disease control group, which included patients with known peripheral neuropathies, demonstrated a higher rate of type I or type

II fibre grouping whereas the samples from ALS patients showed a higher rate of grouping of both fibre types. This suggests that the peripheral neuropathy patients develop a different pattern of denervation without reinervation, which may be why the muscle biopsy appearances of these patients are sometimes distinct from those with ALS.

There are cases where a muscle biopsy may be of diagnostic value in differentiating motor neuron disorders from primary muscle disorders (152). This is especially important in view of the potential for therapeutic intervention in the management of primary muscle disease, where immunomodulatory therapies may be appropriate.

Although some investigators have identified ultrastructural abnormalities of mitochondria in skeletal muscle of patients with ALS (including giant mitochondria, paracrystalline inclusions, and abnormal cristae) (149), there do not appear to be histological characteristics which allow ALS to be differentiated from other degenerative neuropathic conditions. As well as mitochondrial function in muscle tissue, which will be described in greater detail in following sections, work has been performed on muscle fibre function. A study looking at specific force and maximum unloaded shortening velocity in muscle from ALS patients and healthy controls demonstrated that ALS patients had decreased whole muscle specific force but normal single fiber function (153). This would appear to demonstrate that the rate of progression of disease can affect muscle fibre size and function, rather than the presence of ALS, *per se*. This may, however, also be secondary to the denervation that accompanies the disease. A study comparing muscle tissue from patients with ALS and other neurogenic atrophies with normal controls demonstrated increased levels of focal mitochondrial diminution in both disease groups (117). They used the appearance of core-like lesions on NADH staining. This was not looked for specifically in the present study, but the finding concurs with the appearance of certain abnormalities (fibre size variation and fibre angulation in disease and disease control groups compared with normal controls) which have been demonstrated, here. This supports the suggestion that muscle histological changes in ALS are secondary to denervation rather than mitochondrial pathology *per se*.

It is also interesting to note that work has been performed on skeletal muscle energetics, which suggests that an energy imbalance in the muscles of transgenic SOD1 mice may contribute toward a hypermetabolic state, with decreased adipose tissue and elevated metabolic rates (154). Again, difficulties arise in controlling for the pattern of denervation produced by the disease in delineating this as a secondary or primary phenomenon.

The features of muscle biopsy which would strongly suggest mitochondrial disease, such as ragged-red fibres and COX negative fibres (155) were not consistently present in muscle biopsies taken from patients with ALS. This would suggest that while there may be a mitochondrial component to the pathogenesis of ALS, this is not manifest at levels which would affect the structure or function of skeletal muscle tissue.

5 Analysis of Mitochondrial Respiratory Chain Function and Oxidative Damage of Skeletal Muscle from Controls and Patients with ALS

5.1 Aims

To compare the activity of the mitochondrial respiratory chain enzymes in whole muscle tissue taken from patients, controls and disease controls. To compare oxidative damage in muscle tissue by the measurement of aconitase activity in whole muscle homogenates.

5.2 Introduction

Although the primary sites of pathology in ALS are the motor neurons in the spine and brain, a number of investigators have looked at mitochondrial abnormalities in skeletal muscle. As well as the ultrastructural abnormalities, which were dealt with in the preceeding chapter, biochemical mitochondrial function has been assessed.

Mouse models of ALS, that are transgenic for SOD1 mutations, have been used in this field of study. Maximal oxygen consumption and apparent K_m for ADP were decreased in mitochondria from transgenic soleus (an oxidative muscle) (156). SOD activity was demonstrated to be lower in the nervous tissue than the muscle for these mice.

Human skeletal muscle has been investigated for biochemical activity. Response of patient muscle to exercise was found to be abnormal, with increased lactate production, possibly indicative of a respiratory chain disorder (148). The metabolic homeostasis of mice transgenic for SOD1 has been shown to be abnormal with increased energy expenditure and concomitant skeletal muscle hypermetabolism, suggesting that a primary muscle problem may be contributing to the development of the disease (154).

The mitochondrial respiratory chain complex activities have been measured in patients in comparison with controls with spinal muscular atrophy (103). This revealed a decrease in muscle complex I activity in patient samples compared with controls. This was then confirmed using confocal laser-scanning microscopy and video fluorescence microscopy of NAD(P)H and fluorescent flavoproteins (98). Later studies appear to contradict these findings, suggesting that the respiratory function of saponin permeabilised muscle fibres from patients with sporadic ALS is entirely normal (104).

Aconitase has an iron-sulphur cluster $(4FeS)^{2+}$ which is converted to the oxidised form $(3FeS)^{+}$ by superoxide, allowing aconitase activity levels to be used as a secondary assessment of oxidative stress (157). Aconitase activity has been used as a measure of oxidative damage in tissues (158). If the abnormalities in mtDNA which have been identified in patients with ALS (112) are due to an increased level of oxidative damage, then the aconitase levels of the tissue may be affected. In the assessment of aconitase activity from muscle tissue, the aim is to determine whether patients with ALS undergo a greater degree of oxidative damage to all tissues, or whether they are more susceptible to this damage.

In order to establish mitochondrial function in the muscle of ALS patients, the activities of the mitochondrial respiratory chain complexes were measured in mitochondrial fractions and compared with controls. The values were calculated as a ratio to citrate synthase activity. Tissue aconitase activities and respiratory chain complex (I-IV) activities were related to age to determine whether age would affect these values in our sample.

5.3 Approach

Muscle samples were taken from the vastus lateralis muscle as described (section 2.4.1). All samples were taken under a local anaesthetic, transferred to the laboratory in cell culture medium, and frozen down at -80 °C.

For the purposes of analysis, muscle samples were homogenised as described (section 2.9.1). In order to minimise bias and inter-experimental variation, batches of patient, control and disease control samples were processed at the same. The samples were then assigned a code during the analysis which meant that the experiment and subsequent data analysis was performed with blinding to the sample origin. The samples were fractionated as described, and aliquots of the muscle mitochondrial-enriched fraction were snap frozen in liquid nitrogen and stored at -80 °C, until the biochemical analyses were performed.

For the aconitase assays, the spectrophotometric tests were performed on frozen homogenate, and the aliquots were used directly after homogenization and fractionation, without being frozen down, again.

Each assay was performed in triplicate, with an average of the results being used. Protein assays were performed on the aliquots, using the BioRad method (see section 2.9.3). Values for the aconitase assays were calculated as per mg of protein. In order to correct for variations in mitochondrial number, values for the respiratory chain enzyme complexes were calculated as a ratio to total citrate synthase activity (which provides an index of mitochondrial mass) and protein.

A three way ANOVA test was performed on the data to determine whether there was any statistically significant difference between respiratory chain enzyme activities, aconitase activities or citrate synthase activites in control, disease control and patient whole muscle.

5.4 Results

5.4.1 Respiratory Chain Complexes

Spectrophotometric assay of the mitochondrial respiratory chain complexes revealed no significant difference between patients, disease controls and controls in muscle tissue homogenate for any of the enzyme complexes when controlled for citrate

synthase concentration (table 5.1, figure 5.1) and for protein concentration (table 5.2, figure 5.2).

Table 5.1 Average enzyme complex activities in skeletal muscle as a ratio of citrate synthase activity (mean ± SD)

	I	II/III	IV
Controls (n=17)	0.17 ± 0.1	0.17 ± 0.07	0.04 ± 0.016
Disease Controls (n=11)	0.2 ± 0.06	0.2 ± 0.07	0.034 ± 0.014
Patients (n=12)	0.23 ± 0.11	0.19 ± 0.07	0.044 ± 0.014

Table 5.2 Average enzyme complex activities in skeletal muscle as a ratio of sample protein content (mg/ml) (mean ± SD)

	I ($mol.min^{-1}.ml^{-1}$)	II/III ($mol.min^{-1}.ml^{-1}$)	IV ($mol.min^{-1}.ml^{-1}$)
Controls (n=14)	33.10 ± 14.12	33.20 ± 12.59	11.77 ± 4.33
Disease Controls (n=11)	33.60 ± 16.24	33.37 ± 15.14	10.96 ± 4.48
Patients (n=12)	36.53 ± 11.57	33.82 ± 16.04	10.53 ± 5.71

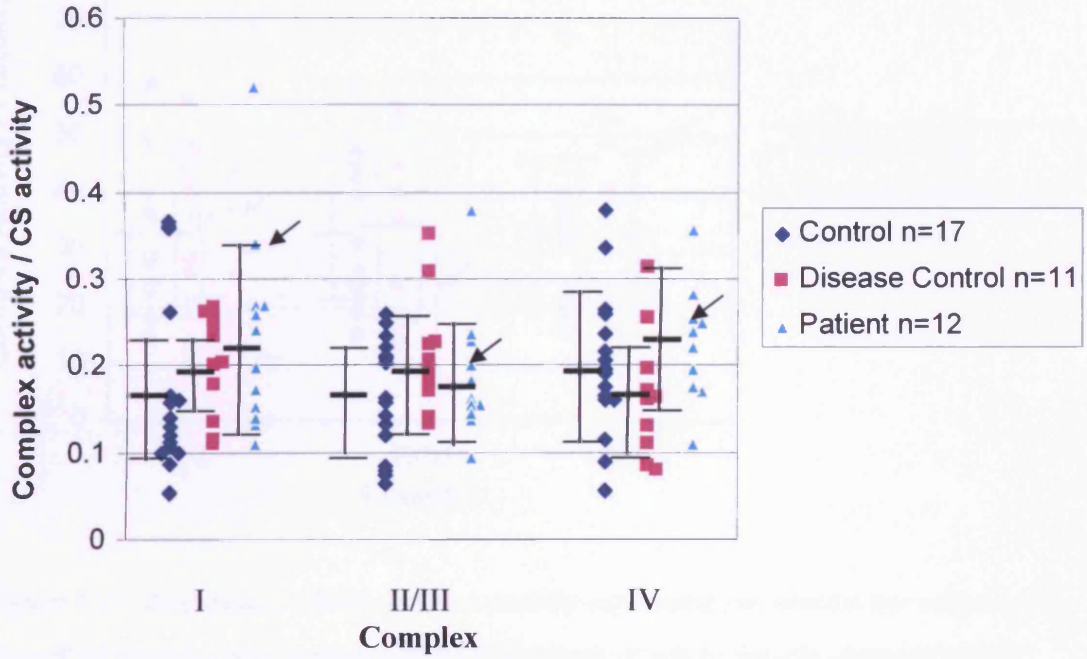


Figure 5.1 – Respiratory chain complex activity per citrate synthase (CS) activity in mitochondrial fractions of whole muscle showing result spread, mean values and SD. The SOD1 mutant sample result (PA11) is indicated (closed arrow). Complex IV results are multiplied by 5 for comparative purposes.

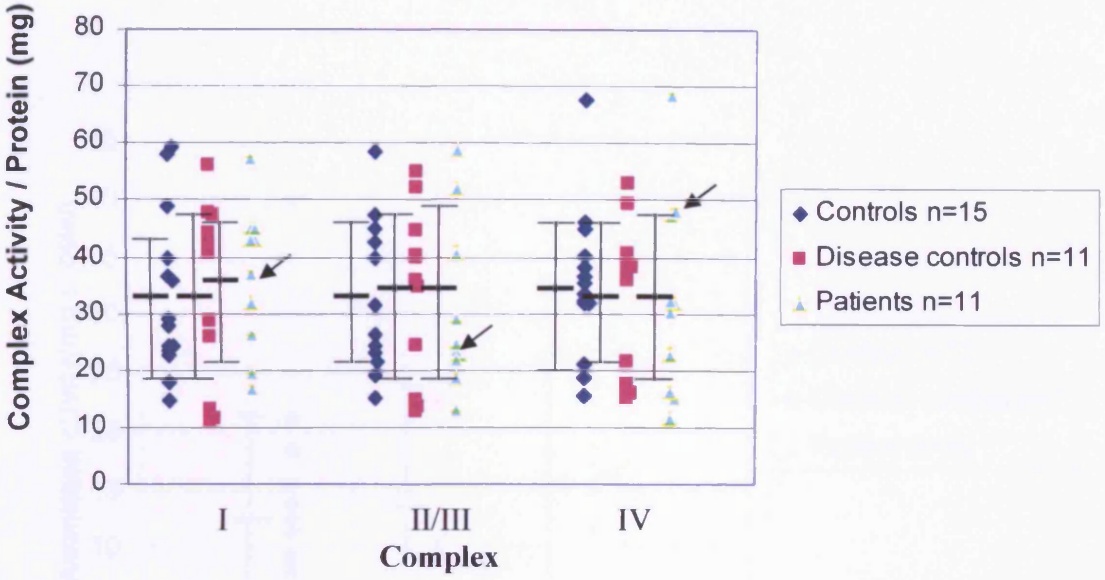


Figure 5.2 – Respiratory chain complex activity expressed per nmoles per minute per amount of protein (mg) in mitochondrial fractions of whole muscle showing result spread, mean values and SD. The SOD1 mutant sample result (PA11) is indicated (closed arrow). Complex IV values are multiplied by 3 for comparative purposes.

5.4.2 Aconitase levels

There was no statistically significant difference in aconitase levels between patient, control and disease control muscle tissue homogenate (table 5.3, figure 5.3).

Table 5.3 Aconitase activity in whole muscle / mg protein

	Mean aconitase Activity / mg protein (U/mg) ± SD
Controls (n=13)	12.96 ± 9.79
Disease Controls (n=7)	16.01 ± 13.00
Patients (n=10)	13.52 ± 10.41

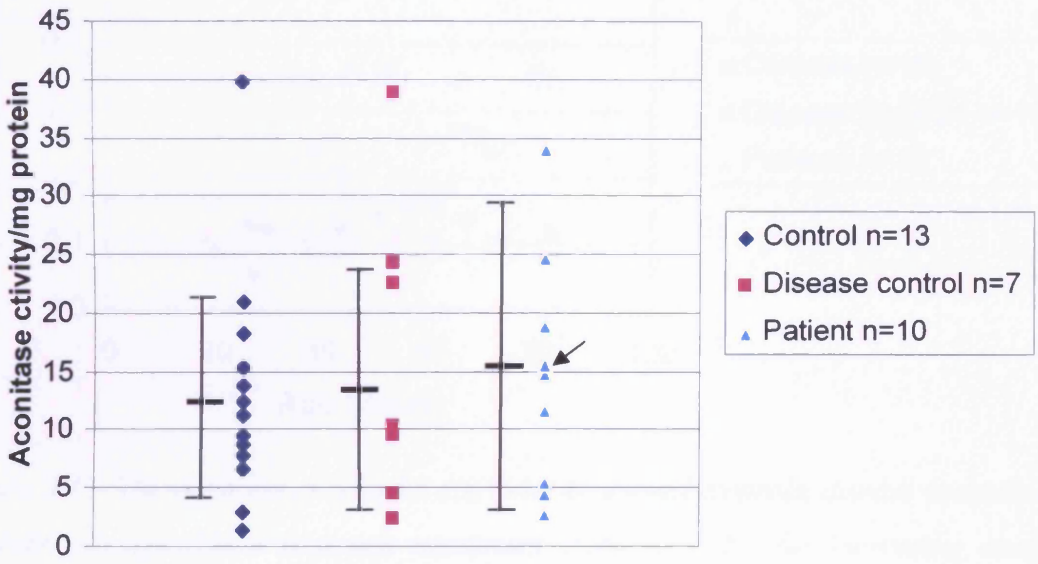


Figure 5.3 – Aconitase activity activity expressed per nmoles per minute per amount of protein (mg) in whole muscle with spread of values, mean and SD. The SOD1 mutant sample result (PA11) is indicated (closed arrow).

5.4.3 Variation in mitochondrial function with age

In order to determine the effect of patient age on mitochondrial respiratory chain function, linear regression analysis was performed for each respiratory chain complex against the age of the patient or control. Because no difference was found between groups, the samples were all pooled to look for variations in complex activity with age *per se*. A trend for increasing activity with age was found for complex I (figure 5.4) ($p=0.07$), but not complex II/III (figure 5.5) ($p=0.54$) or for complex IV (figure 5.6) ($p=0.54$).

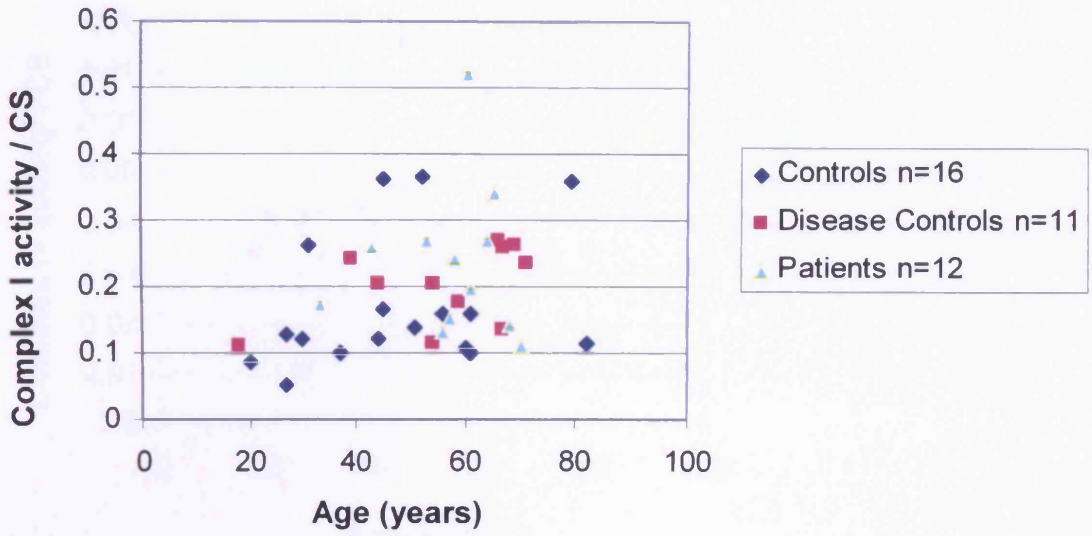


Figure 5.4 – The variation of complex I activity in normal controls, disease controls and patients wih age. There is a non significant trend (p=0.07) for increasing complex activity with age.

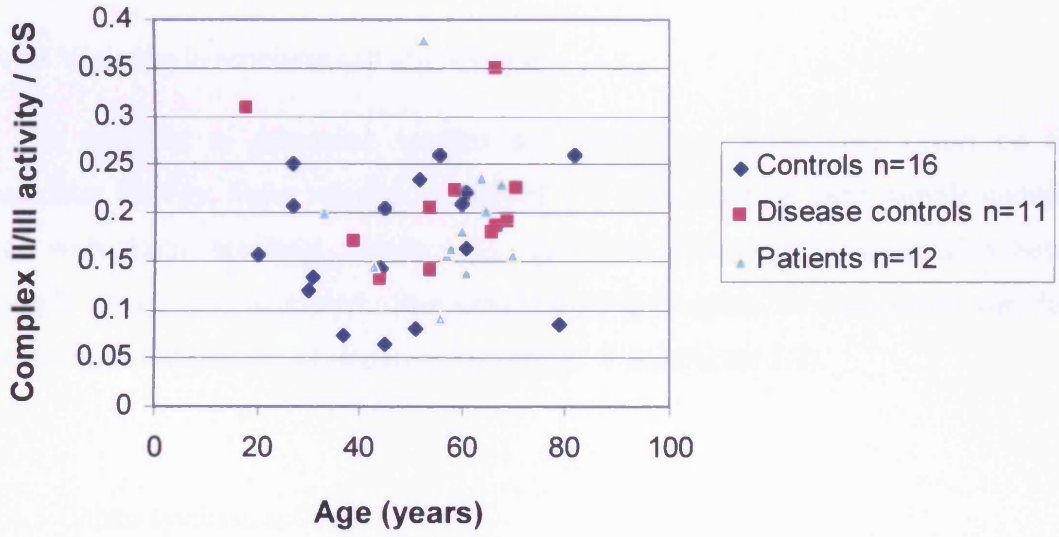


Figure 5.5 – The variation of complex II/III activity in normal controls, disease controls and patients wih age. There is no association.

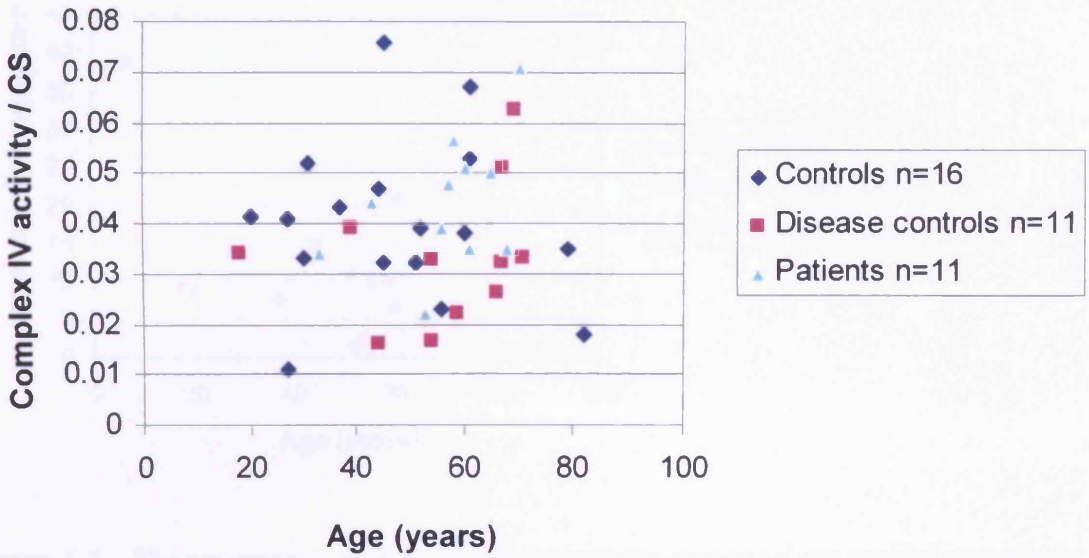


Figure 5.6 – The variation of complex IV activity in normal controls, disease controls and patients wih age. There is no association.

5.4.4 Variation in aconitase activity with age

In order to determine whether age exerted an independent effect on tissue aconitase activity, linear regression analysis was performed for each sample comparing age with tissue aconitase activity. No significant difference in association between sample groups was identified. Analysed as pooled samples, no association was found, indicating similar levels of oxidative damage (p=0.26) (figure 5.7).

5.4.5 Citrate synthase activity

There was no difference in mean citrate synthase activities between the patient, control and disease control muscle samples (p=0.38) (table 5.4, figure 5.8).

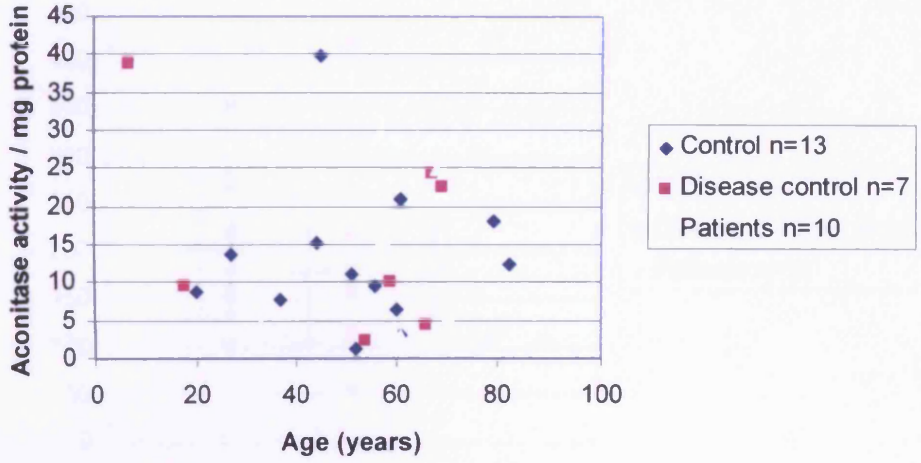


Figure 5.7 – The variation of muscle aconitase activity expressed per nmoles per minute per amount of protein (mg) with age in controls, disease controls and patient samples.

Table 5.4 Citrate synthase activity in whole muscle / mg protein

	Mean aconitase Activity / mg protein (U/mg) ± SD
Controls (n=16)	218.2 ± 84.4
Disease Controls (n=11)	170.9 ± 94.7
Patients (n=11)	173.9 ± 63.3

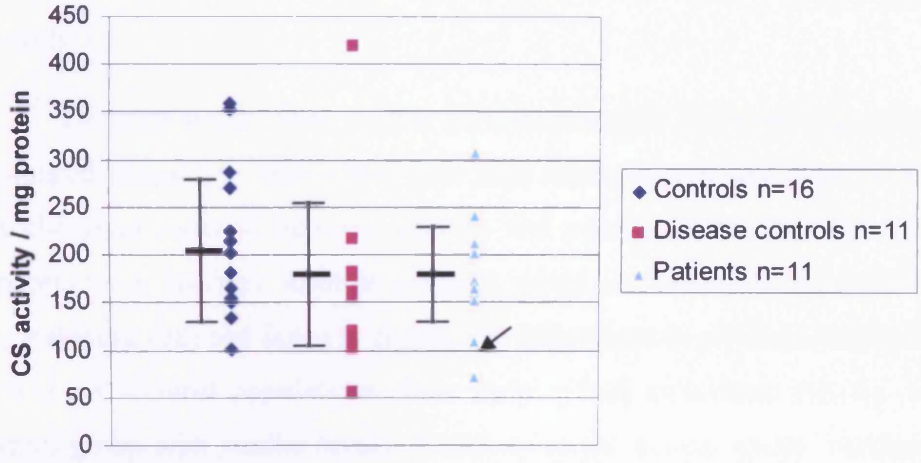


Figure 5.8 – Citrate synthase activity in control, disease control and patient muscle samples per mg of protein showing the spread of values, mean and SD. The SOD1 mutant sample result (PA11) is arrowed.

5.5 Discussion

The study demonstrated that there are no statistically significant functional deficits in skeletal muscle mitochondrial respiratory chain complex activity in patients with ALS compared with normal controls and disease controls.

This finding contradicts that of other work in this field (98), where a specific decrease in complex I activity in the muscle of patients with ALS has been described, although these assays were not controlled for citrate synthase activity and, therefore, the variations in function may represent a variation in the number of functional mitochondria present in the tissue being analysed. This would raise the possibilty that the number or concentration of mitochondria becomes different in muscle tissue from patients with ALS and controls. There is no significant difference between patient and control groups when the samples are controlled for protein content. This would indicate that there is not a general proliferation or decrease in mitochondria secondary to changes in respiratory chain acivity. Taken together, these results suggest that mitochondrial concentration is similar in muscle tissue from patients and controls. This is confirmed by the finding that

citrate synthase activity per mg of protein is the same in patients, controls and disease controls.

Detailed single fibre studies of mitochondrial mediated adenosine diphosphate stimulated respiration rates (104) have also demonstrated that there is no difference in mitochondrial function between patients and controls, however. The data shown here supports these findings. Another criticism raised in this second paper (104) was that the original work (98) had failed to control for differences in physical activity levels between patient and control populations. This study aimed to address this by using a disease control group with similar levels of activity to the patient group. Furthermore, patients and disease controls with similar power in the affected muscles were selected.

A recent study confirms the absence of mitochondrial respiratory chain enzyme abnormalities in muscle tissue taken from patients with ALS when compared with neurogenic (disease) and normal control groups (117). This study looked at enzyme activities relative to sample non-collagen protein content. As a separate finding, mean levels of citrate synthase activity were higher in muscle tissue from ALS patients and disease controls. This implies that enzyme activities controlled for citrate synthase may, in fact, be diminished in ALS and disease control groups. Such an assertion is not supported by our experimental work on citrate synthase activity.

There was no significant difference between aconitase levels in patient and control muscle tissue samples. Aconitase, as a surrogate marker of oxidative damage, may have been affected by the conditions in which the muscle tissue was collected or processed. There was a degree of variability in the time taken from the first incision taken of the muscle, to the time of freezing the sample down in liquid nitrogen, although was within a 2 min period. As it can be assumed that oxidative damage occurs in whole muscle tissue following removal, this may have introduced differences in the aconitase levels which did not accurately affect levels *in vivo*. A similar process may have affected the mitochondrial complex chain activities, although until the muscle biopsy process can be absolutely standardised, it would seem difficult to adequately eliminate this variable.

In view of the fact that the pathological basis of ALS involves damage to the motor neurons, it is perhaps not surprising that there is no difference in levels of oxidative damage seen in peripheral tissues, as there is no reason why patients with ALS would have been exposed to different levels of oxidative stress, and therefore subject to a greater degree of damage, in the remote tissues. If oxidative damage were occuring as a secondary effect of the denervation of the muscle, this was not borne out on analysis of the disease control group, which included patients with peripheral neuropathies and benign fasciculation syndromes. In view of their morphology and function, motor neurons may be subject to greater levels of oxidative stress, and therefore oxidative damage. This would not necessarily result in damage to remote tissues, however. This issue is addressed in a later chapter (9).

The present study identified no relationship between age and respiratory chain activity in muscle mitochondrial fractions. This would appear to contradict some of the earlier studies into the relationship between mitochondrial activity and age. For example, a specific decrease in complexes I and IV (159) and a generalised diminution of all respiratory chain enzymes (160) have been demonstrated in skeletal muscle mitochondria in association with age. However, these early studies have been contradicted by more recent work which demonstrates that when mitochondrial function is controlled for physical activity (such as hand grip strength) rather than age, alone, no relationship between the decline in respiratory chain function and age is seen (161). Smoking status and physical activity, rather than age, were found to correlate with the decline of mitochondrial function in skeletal muscle in another study (162). As none of the patients or controls smoked at the time of our investigation, and as the levels of physical activity were assumed to be similar in the patient and disease control groups, it is, perhaps, not surprising that no clear decline in mitochondrial function in skeletal muscle with age was demonstrated.

6 Analysis of Mitochondrial Respiratory Chain Function in Primary Cultured Cells and Platelet-derived Cybrids from Controls and Patients with ALS

6.1 Aims

To initiate primary myoblast and fibroblast cell cultures and platelet-derived cybrid cell lines from patients with ALS, disease controls with related conditions, and normal controls. To prepare a mitochondrial fraction from these cell lines. To perform biochemical assays in order to establish the presence or absence of a mitochondrial respiratory chain defect in the patient cell cultures.

6.2 Introduction

Mitochondrial dysfunction has been suggested as a significant factor in the aetiology of ALS (102,163,164). There is, for obvious practical reasons, difficulty in obtaining central nervous tissue from patients in the early stages of their disease. The studies that have been carried out on central nervous tissue have, therefore, either been on post mortem tissue (84,97) or on SOD1 mouse models of ALS (165,166). Other researchers have looked at peripheral tissues and cell types including whole muscle (98,117), lymphoblasts (142) and platelets (120-122) in an effort to characterise more general deficits in mitochondrial function that may occur in ALS. Analysis of the activity of the respiratory chain enzymes (figure 1.1) allows an assessment of the primary function of mitochondria to be made, as it is through these metabolic pathways that ATP, and hence cellular energy, is generated.

The assessment of the individual respiratory enzyme complex activities was performed rather than a more generalised "overall" assessment of mitochondrial respiratory function. Different levels of abnormalities of the different complex activities may be able to provide evidence regarding the aetiology of the deficit. This is because while complexes I, III, IV have components coded for by both the mitochondrial and

nuclear DNA, complex II proteins are entirely encoded by the nuclear DNA molecule, with no mitochondrial contribution. While the assessment does provide some evidence regarding the possible aetiology of any deficit that is detected, it should be borne in mind that the assay techniques rely upon the mean mitochondrial activity of thousands of cells, with little information about the intercellular variation of mitochondrial activities within a culture, itself. This problem is adressed in a later chapter.

When reviewing the available evidence for mitochondrial respiratory chain deficiency in remote tissues of patients with ALS, the results are often contradictory. Deficits in fibroblast complex IV activity have been demonstrated in a small number of ALS patient fibroblast cultures (98) (n=3), with normal complex I and complex V activities. Other studies on cultured fibroblasts have shown abnormal superoxide production in mitochondrial fractions and elevated levels of MnSOD (167). Abnormal calcium homeostasis has been demonstrated in the peripheral blood lymphocytes of ALS patients (94). Although this study failed to demonstrate respiratory chain enzyme dysfunction, rate of respiration was demonstrated to be less sensitive to uncouplers in patient lymphocytes.

Human platelets contain mtDNA but no nuclear DNA. Fusion of patient platelets with control cells lacking mtDNA (ρ^0 cells) therefore allows a cell model to be created where the influence of the patient mtDNA can be analysed independently of the context of nuclear expression (124). Using the knowledge that complexes I, III and IV of the mitochondrial respiratory chain have components coded for by the mtDNA, allows conclusions about the aetiology of particular deficits to be drawn from the analysis of cybrid culture enzyme activity. Initial studies suggested that cybrids formed in this way, using the mtDNA of patients with ALS demonstrated deficiencies of complex I activity (120). Subsequent studies have failed to validate this finding, however (121,122). Possible reasons for this discrepancy include the use of different recipient cell lines in the three experiments. The first study utilized SHSY-5Y (neuroblastoma) cells, while one of the subsequent studies used 143B human lung carcinoma lines. These cells are thymidine kinase negative and, therefore, able to survive in BrdU supplemented medium, while nucleated donor cells would be selected against. This raises the possibility that the

original study (120) results may have been distorted by nucleated cell contamination. The levels of oxidative metabolism undergone by the two cell lines have been shown to be similar (123), implying that the difference in respiratory chain activity was not due to differences in the recipient cell metabolic profiles.

6.3 Approach

6.3.1 Sample Generation

Primary cultures of myoblasts, fibroblasts and platelet-derived cybrids were initiated as described (section 2.2). These cultures were grown up on 10-cm plates in order to obtain a sufficient number for production of a mitochondrial fraction appropriate for functional assays. For myoblasts and fibroblasts, 20 confluent 10-cm plates of cells were harvested. For cybrid cultures, 8 confluent 10-cm plates were harvested. Mitochondrial fractions were prepared from the cell cultures as described (section 2.5.5). The passage number of all cell cultures at harvesting was between 4 and 6. Unfortunately a number of primary myoblast and fibroblast were lost to fungal infection. Some fibroblast cultures failed to seed from the initial skin sample (table 6.1). Although this may have been due to the agents used to clean the skin prior to biopsy, the surgeons all maintained that they used an equivalent amount of betadine for this purpose.

In order that samples with oxidative deficits were not selected against, the growth medium was supplemented with pyruvate and uridine, as described (124).

6.3.2 Cybrid Analysis

Experimental A549 (human lung carcinoma) ρ^0 cells were used to make the platelet-derived cybrids. In order to determine successful cybrid formation, immunocytochemical staining was performed on the cells labelled with BrdU. This is a thymine nucleoside analogue which is actively incorporated into replicating DNA and can be detected with a fluorescent labeled antibody to reveal areas of replicating DNA.

Therefore in cells without mtDNA, only nuclear staining should be visible, whereas in ρ^0 cells repleted with mtDNA, there should be staining outside the nucleus within the cytoplasm (168). This was confirmed for each cybrid culture (figure 6.4).

In a further confirmation of the successful incorporation of wild type platelet DNA into the experimental cybrid cultures, a restriction enzyme digest was performed on a PCR-amplified mtDNA fragment from the cybrid cultures in order to exclude the persistence of the A549 mtDNA in cultures. *AluI* is a restriction enzyme which cuts TC/GA sites. A549 mtDNA has a 2 bp deletion of a CA repeat compared with wild type mtDNA (figure 6.1) at position 514-523. By using a forward primer (M60 – see 2.5.7) and a specific mismatch reverse primer in a PCR of this section of mtDNA, a Taq I site was generated when A549 mtDNA was amplified, but not when wild-type mtDNA was amplified (figure 6.1).

Nucleotide position: 509 535

Wild type mt DNA: 5'-CCCAGCACACACACCGCTGCTAACC-3'

Primer (M61): GAGTGTGTGGCGACGATTGG-5'

A549 mtDNA: 5'-CCCAGCACACACACCGCTGCTAACC-3'

....after amplification: CCCAGCTCACACACCGCTGCTAACC-3'

Figure 6.1 Mismatch PCR of mtDNA to generate an AluI digest site in mtDNA from A549 mtDNA. The mismatching nucleotide of primer M61 and Taq I restriction sites are underlined.

The products containing the polymorphism should generate a 146-bp digestion product, whereas the wild type should remain at 164-bp, as it should lack the Taq I cleavage site. The products of the digest were run on a 4% agarose gel with a ϕX174 DNA/*HinfI* marker. Again, this was confirmed for each culture (figure 6.5).

6.3.3 Assays

In order to minimise error due to variablility in experimental conditions between assays, cultures of control and patient-derived cells were grown and harvested at the same time. The mitochondrial fraction was prepared from each at the same time. In order to minimise experimental bias, the mitochondrial fractions were assigned a code by an independent observer, so that the assays would be performed "blind" as to the origin of the mitochondrial fraction. The samples were subsequently decoded when the assays and post-experimental data processing had been carried out.

Separate assays were performed for complex I (NADH-Coenzyme Q reductase), complex II/III (succinate cytochrome c reductase) and complex IV (cytochrome c oxidase) activity (140-142,169). In order to control for variations in mitochondrial concentration between samples, the samples were also assayed for citrate synthase activity, and the final enzyme activity was expressed as a ratio of activity per citrate synthase activity. All assays were performed at 30°C. Three assays were performed for each complex on each sample, and the final activity was given as an average of the three values.

Enzyme activities in muscle and cultured myoblasts were compared using linear regression analysis to determine whether there was a correlation, and hence persistence of possible mitochondrial deficits in cultured cells.

A three way ANOVA test was performed on the data from the patient, normal control and disease control samples to detect any statistically significant difference in enzyme activity.

6.4 Results

Assays were performed on mitochondrial fractions from ALS patients, normal controls and disease controls as described (section 2.8). The numbers of fractions available are given for each cell type (table 6.1)

Table 6.1 Cell culture numbers assayed for respiratory chain enzyme complex activity

Control / patient	Cell type	Number of samples
Controls	Myoblasts	10
	Fibroblasts	8
	Cybrids	6
Disease Controls	Myoblasts	7
	Fibroblasts	5
	Cybrids	3
Patients	Myoblasts	10
	Fibroblasts	8
	Cybrids	6

6.4.1 Myoblasts

There was no significant difference in respiratory chain complex activity between patient, control and disease control cultured myoblasts. Although the mean complex IV activity appeared markedly lower in the patient samples compared to the control samples, this difference was not significant ($p=0.23$). (Table 6.2, Figure 6.2).

Table 6.2 Mean respiratory chain assay activities relative to citrate synthase activities for myoblasts ± SD

Cell Culture Type	Complex I	Complex II/III	Complex IV*
Control	0.17 ± 0.07	0.29 ± 0.1	0.048 ± 0.020
Patient	0.15 ± 0.07	0.26 ± 0.11	0.032 ± 0.016
Disease Control	0.17 ± 0.08	0.29 ± 0.05	0.036 ± 0.028

6.4.2 Fibroblasts

There was no significant difference in any of the respiratory chain enzyme complex activities measured for patient, disease control and normal control fibroblasts (table 6.3, figure 6.3).

Table 6.3 Mean respiratory chain assay activities relative to citrate synthase activities for fibroblasts ± SD

Cell Culture Type	Complex I	Complex II/III	Complex IV
Control	0.16 ± 0.08	0.23 ± 0.08	0.042 ± 0.020
Patient	0.13 ± 0.06	0.25 ± 0.07	0.052 ± 0.018
Disease Control	0.23 ± 0.09	0.25 ± 0.04	0.038 ± 0.024

6.4.3 Cybrids

The successful incorporation of platelet DNA from patients and controls to generate experimental cybrids was confirmed by BrdU staining (figure 6.4) on the cybrid cell lines generated. PCR of cybrid DNA followed by *AluI* digestion and agarose gel electrophoresis was performed on the cybrid cultures, and confirmed that the mtDNA incorporated into the cybrids was wild type (figure 6.5). Only 3 disease control cultures of platelet cybrids were successfully grown. Complex IV activity was found to be lower

in disease controls than in patient cybrids (p=0.084). Otherwise, there was no significant difference in respiratory complex activity between patient, control and disease control cultured cybrids (table 6.4, figure 6.6).

Table 6.4 Mean respiratory chain assay activities relative to citrate synthase activities for platelet-derived cybrids ± SD

Cell Culture Type	Complex I	Complex II/III	Complex IV
Control	0.31 ± 0.11	0.3 ± 0.07	0.064 ± 0.026
Patient	0.31 ± 0.17	0.36 ± 0.11	0.060 ± 0.020
Disease Control	0.36 ± 0.19	0.29 ± 0.06	0.036 ± 0.006

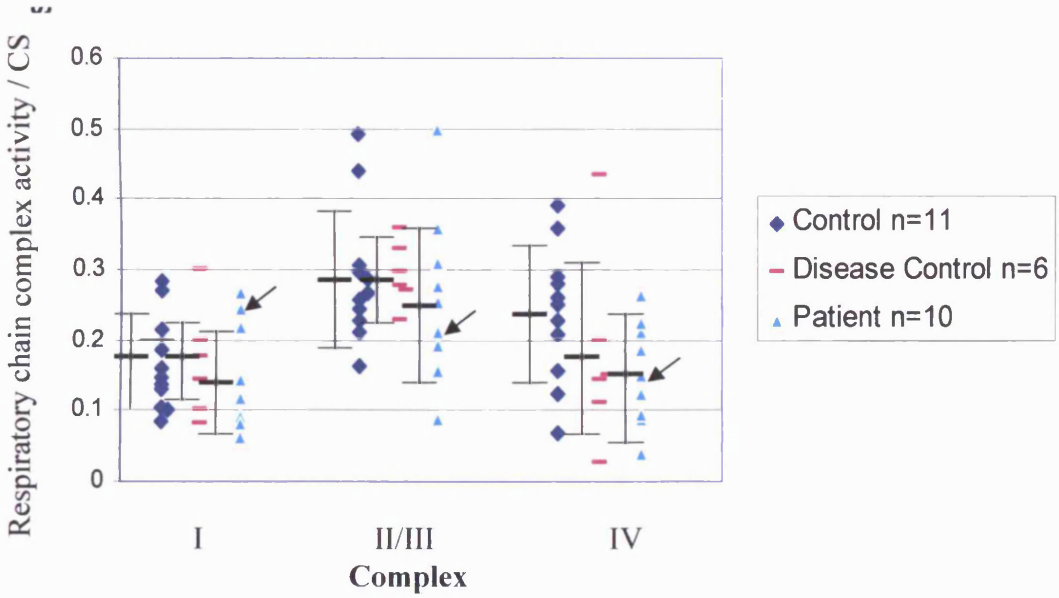


Figure 6.2 Mitochondrial respiratory chain activities relative to citrate synthase (CS) activity in cultured human myoblasts showing spread of values, mean and SD. The SOD1 mtant sample result (PA11) is arrowed. Complex IV activities are multiplied by 5 for comparative purposes

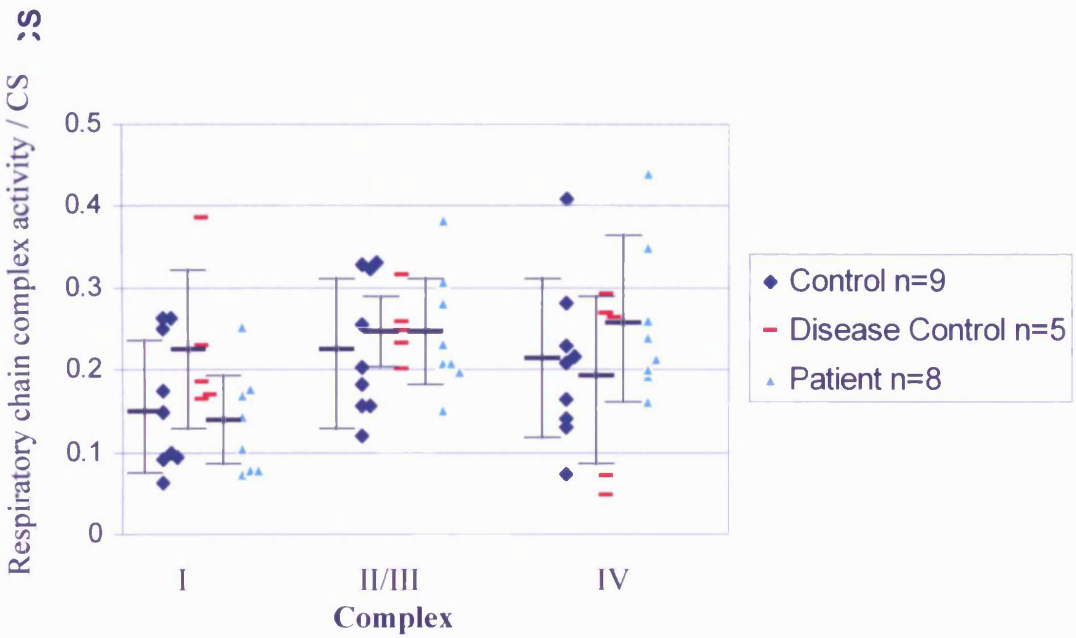
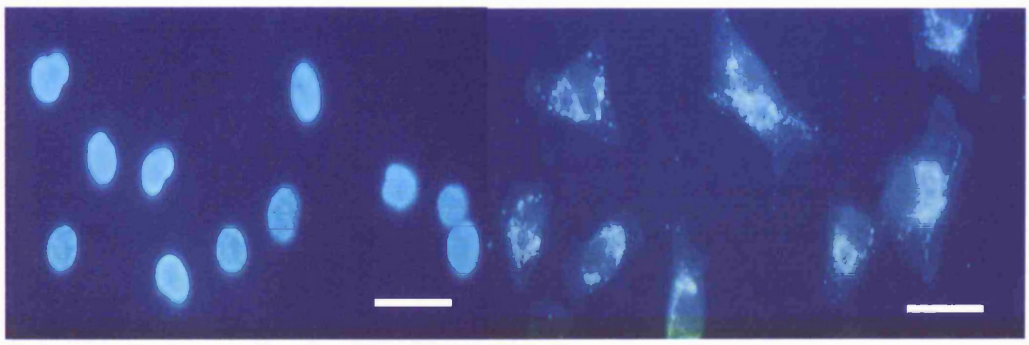


Figure 6.3 Mitochondrial respiratory chain activities relative to citrate synthase (CS) activity in cultured human fibroblasts showing spread of values, mean and SD. Complex IV activities are multiplied by 5 for comparative purposes.



a b

Figure 6.4 Bromodeoxyuridine staining of replicating DNA. Staining of ρ^0 A549 cells (a) and platelet fusion cybrids from control subject C11 (b) demonstrates the absence of mtDNA in the ρ^0 cells, and the presence of mtDNA in the cybrid cells. Bar=20μm.

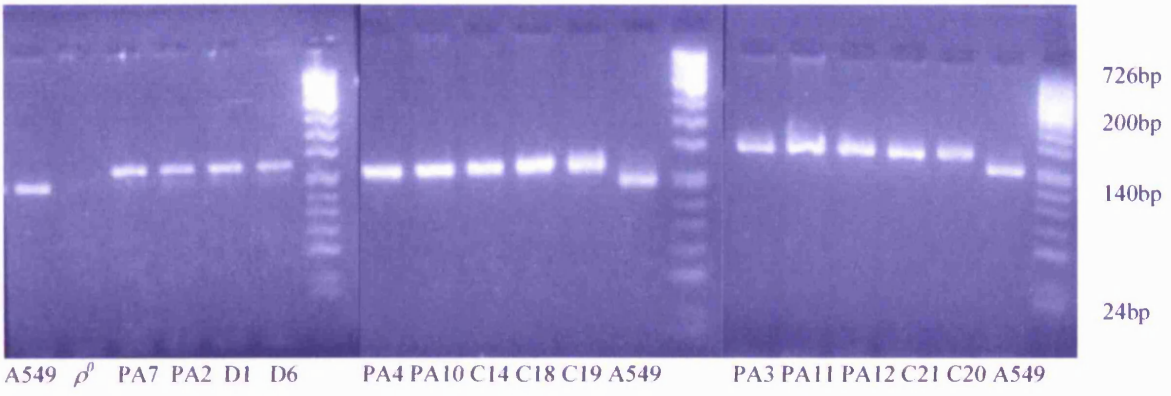


Figure 6.5 Ethidium bromide stained agarose gels showing AluI digested PCR products of the mtDNA of cybrids and parental A549 cells. The experimental cybrids lack the digestion site, indicating an absence of parental A549 mtDNA. No PCR product was detected with DNA extracted from A549 ρ^0 cells.

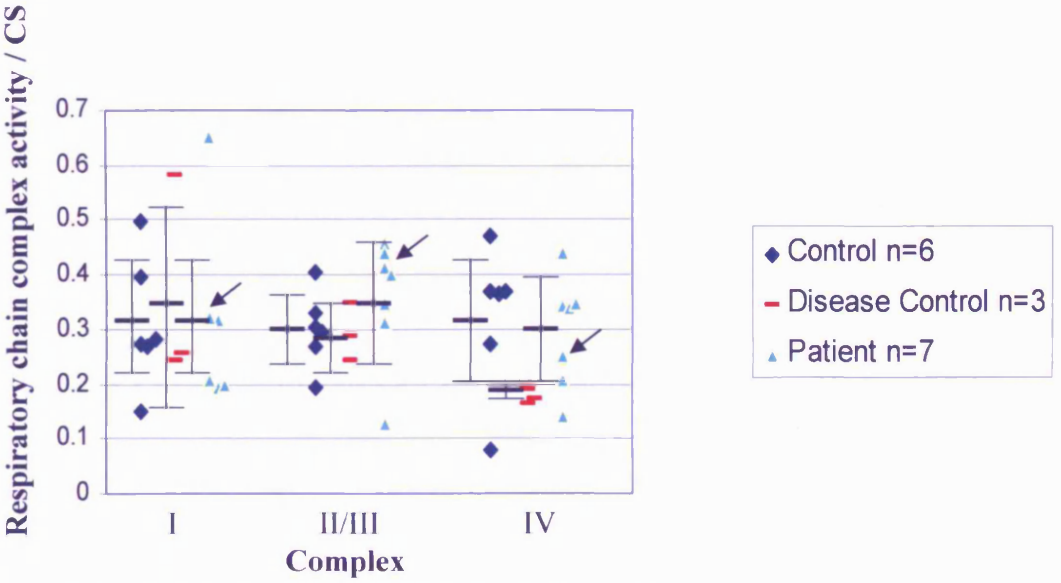


Figure 6.6 Mitochondrial respiratory chain activities relative to citrate synthase(CS) activity in platelet-derived cybrids showing spread of values, mean and SD. The SOD1 mutant sample result (PA11) is arrowed. Complex IV values are multiplied by 5 for comparative purposes.

6.4.4 Variation of mitochondrial function with age

In order to determine the effect of age on mitochondrial respiratory chain function, linear regression analysis was performed for pooled cell samples' respiratory chain complex activity against the age of the patient or control. Fibroblast complex IV results showed a negative association with increasing age, although this did not quite reach significance. There was no other relationship between biological age and respiratory chain function demonstrated.

Table 6.5 The statistical significance of the association between age and mitochondrial respiratory complex activity relative to CS activity for different cell cultures.

Cell culture complex analysed	**P value**
Myoblast complex I	0.13
Myoblast complex II/III	0.43
Myoblast complex IV	0.46
Fibroblast complex I	0.56
Fibroblast complex II/III	0.23
Fibroblast complex IV	0.05
Cybrids complex I	0.54
Cybrids complex II/III	0.90
Cybrids complex IV	0.41

6.4.5 Correlation of muscle tissue and myoblast assays

Linear regression analysis was performed on the different sample results to determine the relationhips between respiratory chain complex activity (controlled for citrate synthase activity) in the different tissue and cell types for the same (pooled) samples.

There was a strong correlation between the muscle samples and the myoblast cultures derived from a particular sample for complex I ($p=0.03$) (figure 6.7).

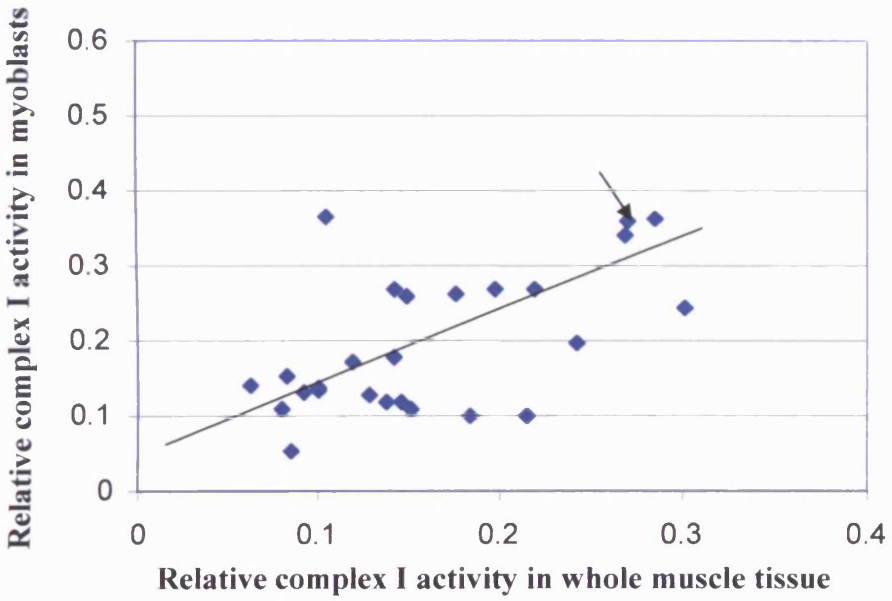


Figure 6.7 Complex I activity (activity/CS) in whole muscle tissue and cultured Myoblasts derived from the muscle. The SOD1 mutant sample result (PA11) is arrowed.

Complex II/III showed no correlation (p=0.6) between activity in muscle tissue and cultured myoblasts, while complex IV showed a trend but no statistical correlation (p=0.14).

6.5 Discussion

The initiation of primary cell cultures from tissue and the generation of platelet-derived cybrids was not always successful. Around half of the attempted cybrid fusions of mtDNA from platelets with ρ^0 cells failed. A significant proportion (around half) of fibroblast cultures failed to seed from the initial skin biopsy specimens, while some myoblast samples were lost to fungal infection and incubator break down during the cell culture process (15 out of 44). The amount and type of skin disinfectant used in the surgical excision of the skin biopsy may have influenced the ability of fibroblast cultures to seed from the primary tissue, although this was generally felt to be consistent. The

orientation of the pieces of tissue on the 10-cm culture plates may also have influenced the success of the culture initiation.

The spectrophotometric assays themselves produced a wide spread of results around the mean for each sample. This is, perhaps, surprising given that the assays involve the analysis of a cell homogenate. However, this phenomenon has been seen in previous studies of respiratory chain activity assays in cultured cells using similar techniques (103,122,170).

Although differences in respiratory chain activity between patients with ALS and controls have been identified by others in muscle tissue (98,103), these studies have not been previously performed on cultured myoblasts. The present study identified no differences in activity between cultures taken from patients with ALS, normal controls and disease controls. There are a number of possible explainations for this. It may suggest there are, indeed, no differences or that mitochondrial function in the muscle of ALS patients is affected by the disease process itself, rather than as the consequence of an intrinsic abnormality of the mitochondria. Alternatively, if qualitative mitochondrial deficiencies occur as the result of random intercellular mosaicism, these effects may be attenuated in succeeding generations of cultured cells. This would not appear to be a significant factor in the assessments performed, because the cell cultures were all analysed at the same passage numbers. To address this issue, the same biochemical assays were performed on whole muscle tissue. These experiments are detailed above (chapter 4). It is also worth pointing out that the findings of mitochondrial abnormalities from published studies on mitochondrial respiratory chain function in patients with ALS are often conflicting. In one of the first papers to identify a difference in mitochondrial respiratory chain activity (98), complex IV was determined to be normal in muscle tissue taken from patients, and attenuated in fibroblasts from the patient group. Complex I activity, however, was diminished in patient muscle tissue, but normal in cultured fibroblasts. These results would suggest that a process of greater complexity than primary deficits in mitochondrial expression may be occuring. It may be that a greater number of samples utilised in the current study would have been able to demonstrate small

differences between ALS patients and control groups in terms of mitochondrial respiratory chain activity.

The results of the assays performed suggest that age-related changes in mitochondrial function are not of sufficient magnitude to produce a decrement in the levels of activity of the mitochondrial respiratory chain enzymes in cultured cells. A number of studies have found that ageing is associated with impairment in oxidative phosphorylation and damage to the mtDNA (43,171,172). There are no consistent discrete complex abnormalities associated with ageing, however (160), and this is borne out by the results of these biochemical assays. Fibroblasts have been shown to develop abnormalities of mtDNA with ageing (173), and the presence of a negative association between complex IV activity (with mtDNA coded components) and age suggests that some functional impairment may occur as a result of acquired abnormalities of the mtDNA. A decline in complex IV function with age has been described by one study, although this used skeletal muscle (44). As this association was not seen in myoblasts or cybrids and it would be interesting to speculate as to whether acquired mtDNA abnormalities in fibroblasts occur as the result of solar damage to the skin (174). Indeed, mtDNA deletion levels have been described as a biomarker for sun exposure in normal skin (175).

The normal results for platelet respiratory chain activity found in SALS patients were also seen in a previous cybrid study which utilised platelets (122), while direct analyis of platelets from patients with SALS revealed no abnormalities in cytochrome oxidase activity or glutamate metabolism (121). Platelet incorporation, from patients into ρ^0 cells does not, therefore, seem to produce consistent deficits in mitochondrial function. These results do seem to conflict with the results of another group (120), but as alluded to in the introduction, contamination with nucleated cells cannot be excluded in this study.

It is interesting that there is a strong correlation in enzyme complex activities between muscle and myoblasts for complex I and less so for the other complexes studied. This would imply that whereas expression of these complexes may change over time in cell cultures, the expression of complex I appears to be relatively preserved.

7 Immunocytochemical Assessment of Mitochondrial Protein Expression in Cell Cultures from Controls and Patients with ALS

7.1 Aims

To determine whether the intercellular expression of the mtDNA-encoded subunit I of cytochrome c oxidase (complex IV) protein is different in cultured cells from patients with ALS compared to controls.

7.2 Introduction

Subunit I of complex IV of the electron transport chain (COX I) is encoded for by mtDNA (figure 1.1). Quantification of the expression of this protein allows an assessment to be made of the integrity of mtDNA expression. It would be predicted that significant abnormalities of mtDNA would produce abnormalities in the expression of mtDNA-encoded proteins. In a population of cells where mtDNA abnormalities may vary between cells it is important to be able to look at the protein expression of individual cells, as a population assay would mask intercellular differences. A threshold effect whereby a cumulative number of mutated mtDNA molecules would need to be present to produce a generalised abnormality of mitochondrial function applies. Many mitochondrial disorders demonstrate mtDNA heteroplasmy (the presence of more than one type of mtDNA within cells) at a cellular level (176), with a large degree of intercellular variation in mtDNA abnormalities. The biochemical assays used to measure specific enzyme complex activities look at the gross average for a population of cells. If there were discrete random mtDNA abnormalities throughout the sample that were present in only a few cells, then it would be expected that these would not contribute to a demonstrable abnormality in an assay of a pooled cell sample. Furthermore, the spread of results in the cellular biochemical assays was large (chapter 6), implying that there is much variation between samples, even before consideration of any potential heteroplasmy of mtDNA.

Acquired abnormalities of the mtDNA molecule may only be present in a small proportion of the cultured cells being studied. Even with appropriately supplemented (pyruvate and uridine) growth medium (124) this may become less pronounced with increasing passage number, whereby lines of cells which have developed mtDNA abnormalities are selected against and become depleted. For this reason, it is important to compare control and patient cell lines at equivalent passage numbers for phenotypic analyses.

7.3 Approach

The presence of a green fluorescent anti-COX I antibody was used as an indicator of COX I expression in the cell. The amount of COX I per cell will be dependent upon the number of mitochondria present within the cell. In order to control for variations in the number of mitochondria per cell, cells were treated with a red fluorescent dye (Mitotracker), which is selectively taken up by the mitochondria. The ratio of the signals of the antibody and the Mitotracker were measured for each cell and the ratios found in patient cells were compared with those found in control cultures.

Cultures of fibroblasts and myoblasts were initiated from primary tissue samples as described (section 2.3). At the same passage number (between 2 and 5), the cells were put onto glass cover slips at a medium density and left to grow for 24 hours. The cells were stained with Mitotracker dye (see paragraph 2.6.2).

The cells were then fixed and stained. The method involved incubation of the fixed cell culture on the cover slip with a mouse monoclonal antibody against subunit I of complex IV (COX I), followed by immunofluorescent detection of the primary antibody with green fluorescent (Alexa 488) labelled secondary antibody. The cell nuclei were stained blue with 4',6-diamidino-2-phenylindole (DAPI). Mr J Muddle carried out the cellular analysis of the stained cells; the slides were examined with a Zeiss Axiophot microscope fitted with a computer controlled motorised stage and a triple band pass emission filter. The three separate fluorescent images, red, green and blue, were captured using an automatic integrated camera system (figure 7.1). The system scanned 100 fields

in a 10 x 10 matrix. The three integrated images (green, red and blue) were recorded for each field position. The fluorescent blue nuclear image (DAPI) was used to define the location of each cell, while the integrated images defined cell boundaries and produced a binary mask image of each of the mitochondria. Deficiencies in expression of the respiratory chain complex would be reflected in decreased green immunostaining with the red Mitotracker staining level remaining constant. Densitometric measurements of grey levels made for red and green images allowed the proportion of cells deficient in a particular complex to be quantified (figure 7.2).

Myoblasts were analysed in experiments where one patient would be matched with a disease control and a normal control, stained and fixed and analysed at the same time. Fibroblasts were pooled, so that all patient, control and disease control samples were stained, fixed and analysed at the same time. This allowed the total cell populations of the samples to be compared.

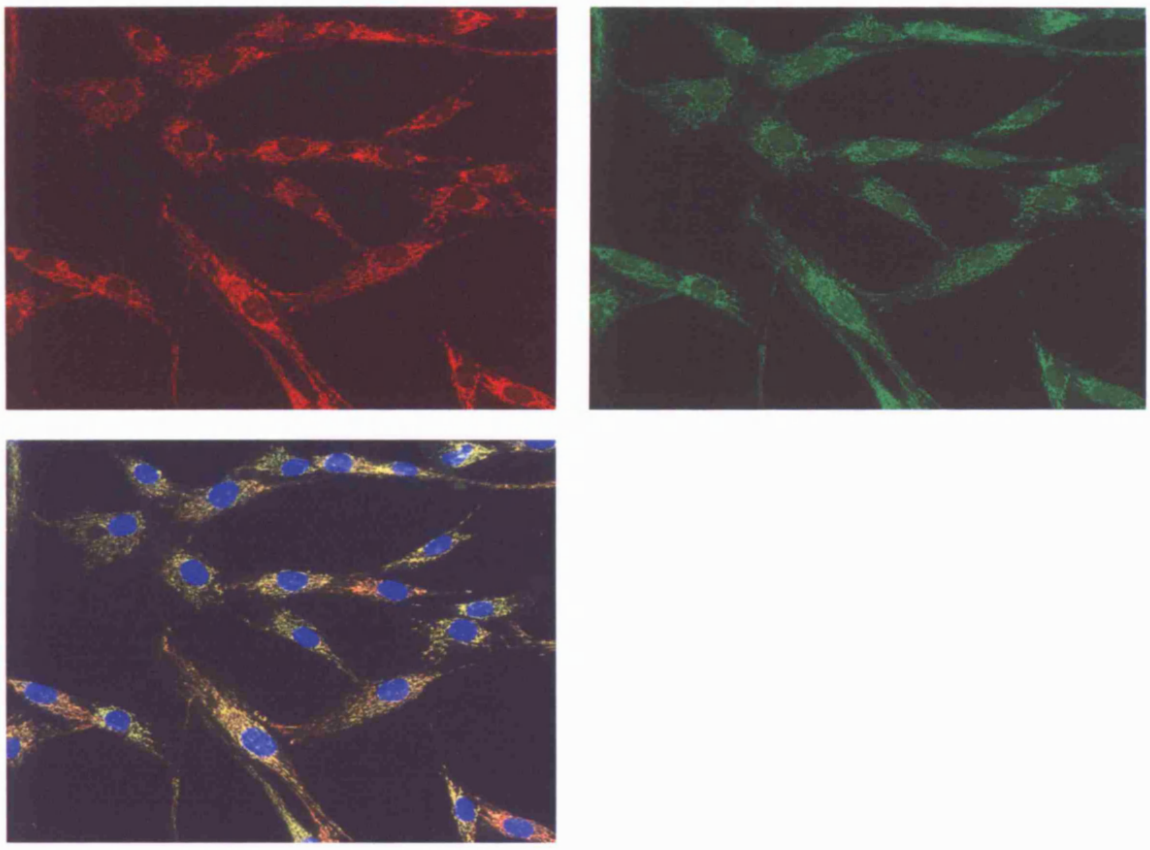


Figure 7.1 Cultured myoblasts stained with (clockwise from top left), Mitotracker, green labelled anti-COX I and colocalisation with DAPI staining of the nucleus.

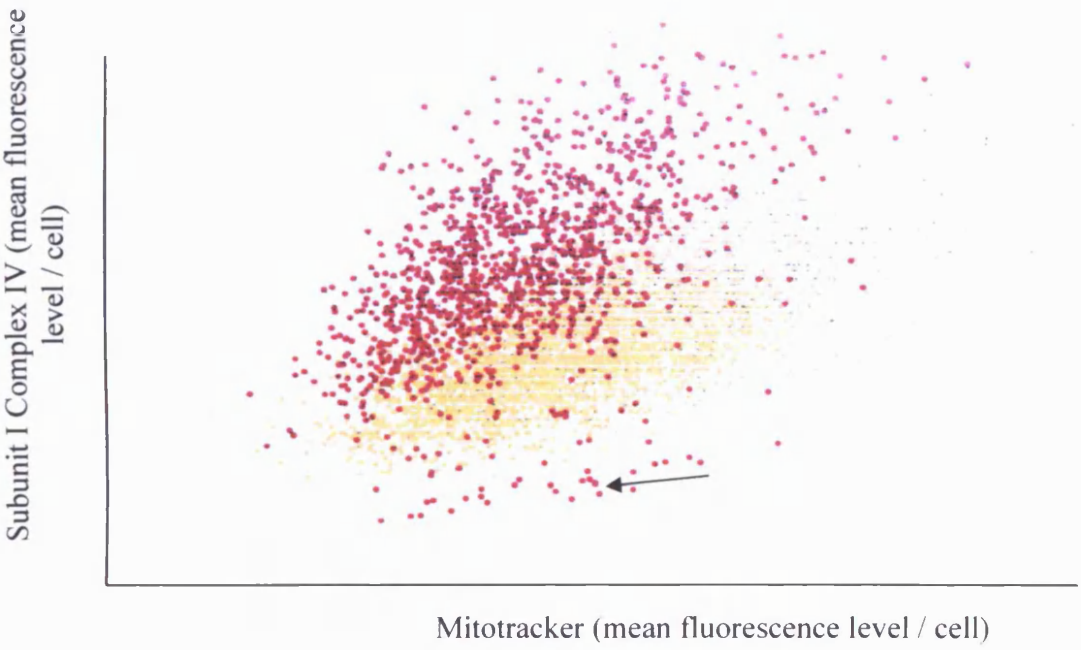


Figure 7.2 Levels of complex IV subunit I per cell plotted against mean Mitotracker levels in the fibroblasts of a control subject (yellow dots) and a patient with known mtDNA depletion (red dots). There is a small sub-population of patient cells which are seen to demonstrate low levels of subunit I expression (arrowed).

7.4 Results

Myoblasts were initially analysed on an individual age- and sex-matched basis to look for diifferences in distribution of COX I staining relative to Mitotracker between the cells of individual samples. Because of technical considerations and inter-experimental variation, the results for single patient / control analyses were not completed sufficiently to provide useful data. Because the cells were being looked at on an individual basis, the cell cultures samples were pooled into patient and control groups. Fibroblast samples C5, C6, C7, C8, C14, C17, C18, C21, D3, D4, D6, D7, D8, PA1, PA2, PA3, PA4, PA6, PA8, PA9, PA10, PA11, PA12 were analysed together. For fibroblast cultures, there was no difference between pooled patient and pooled disease control and control cells in terms of levels of COX1 expression (figure 7.3), Mitotracker expression (figure 7.4) or in the ratio of subunit I expression to Mitotracker (figure 7.5). It is important to note that the uptake

of Mitotracker is the same in the control and patient cell populations. If there was a major difference in Mitotracker staining between the cell populations, then it would be impossible to form conclusions about the expression of COX subunit I, as this forms the basis for the ratios.

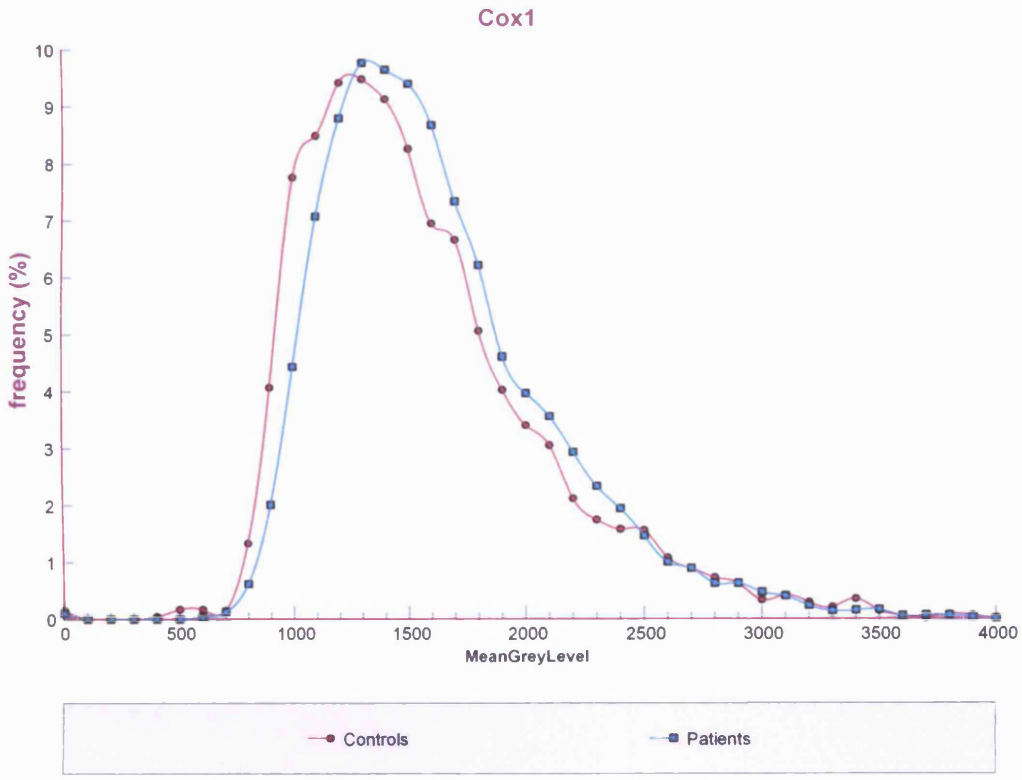


Figure 7.3 Line graph demonstrating the mean levels of COX I expression per cell (fibroblasts) for the total cell population analysed. Total number of cells is 100%. Disease control and control cells are pooled. (Total patient cells n=8 x 10^3, total control cells n=10 x 10^3).

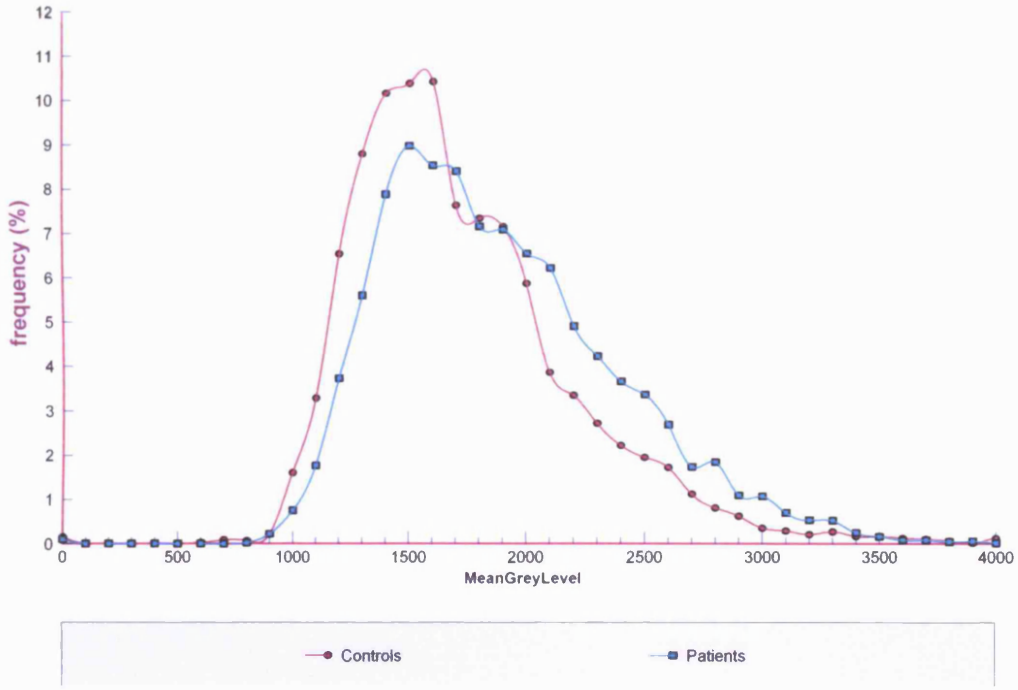


Figure 7.4 Line graph demonstrating the mean levels of Mitoracker expression per cell (fibroblasts) for the total cell population analysed. Total number of cells is 100%. Disease control and control cells are pooled. (Total patient cells n=8 x 10^3, total control cells n=10 x 10^3).

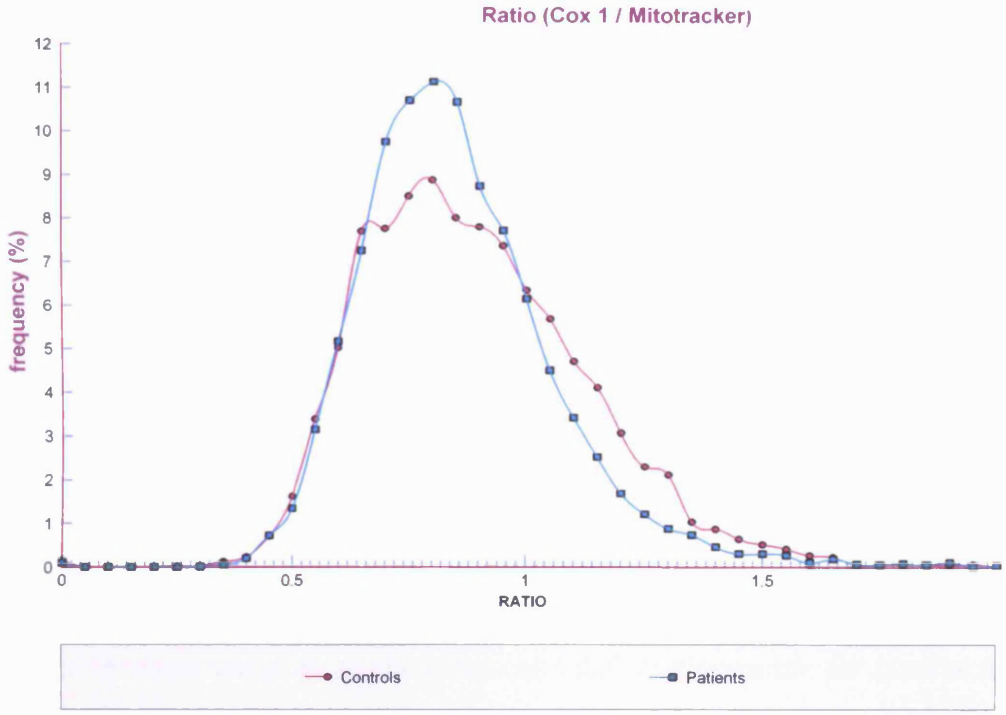


Figure 7.5 Line graph demonstrating the ratio of COX 1 expression over Mitotracker expression per cell (fibroblasts) for the total cell population analysed. Total number of cells is 100%. Disease control and control cells are pooled. (Total patient cells n=8 x 10^3, total control cells n=10 x 10^3).

When looking at each cell individually, there was not an apparent sub-group of cells amongst the patient population which were seen to be deficient in COX1 expression relative to Mitotracker (figure 7.6).

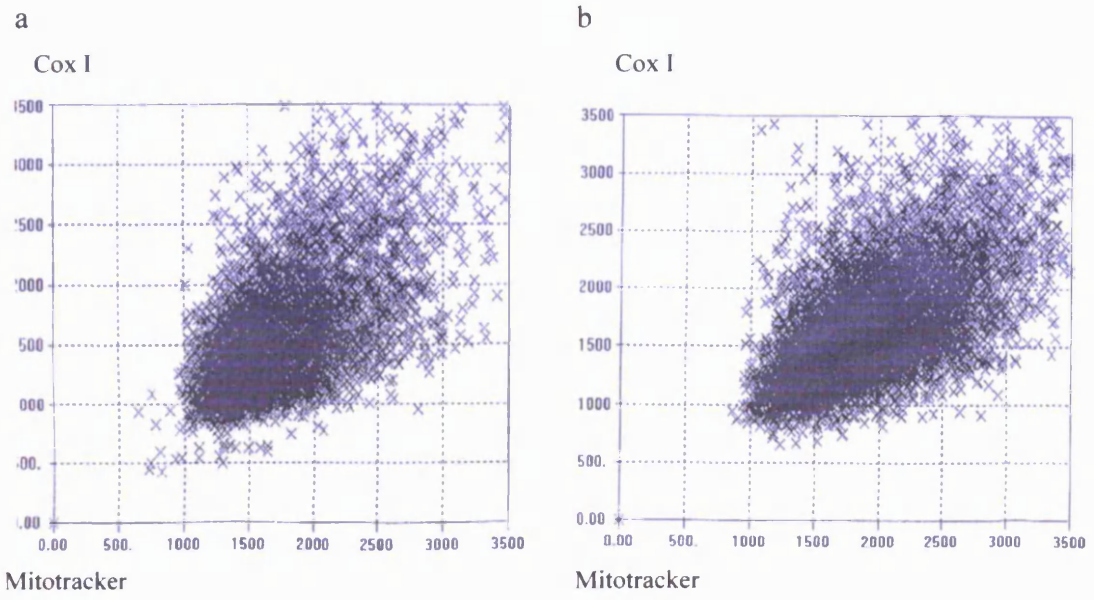


Figure 7.6 A cloud diagram of the ratios of COX 1:Mitotracker for patient (a) and control (b) fibroblast cell cultures. (Total patient cells n=8 x 10^3, total control cells n=10 x 10^3).

COX subunit I negative cells, if present, would be visualised, as for figure 7.2, with a distinct sub-population below the main body of cells on the scatter plot. When looked at individually, these cells would show red flourescence with reduced green counter staining (figure 7.7). There were one or two COX negative cells in some of the control and patient cell samples, but there were no differences in the number of these seen between sample types.

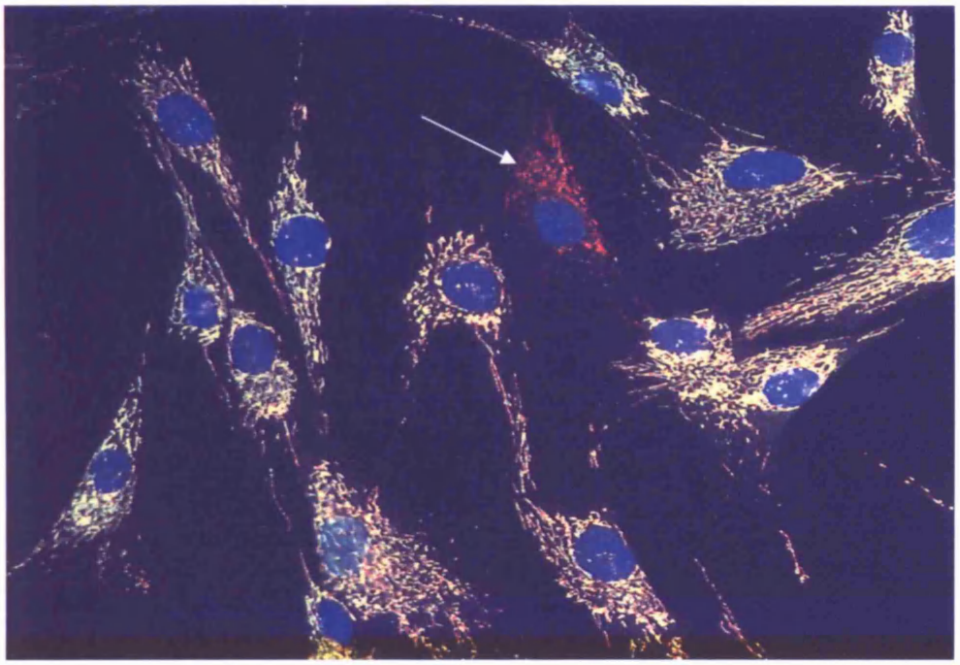


Figure 7.7 PA9 fibroblasts counterstained with Mitotracker, anti-COX I and DAPI, demonstrating a COX subunit I negative cell (arrowed) which has visible red staining from the Miotracker, but no green COX I staining.

7.5 Discussion

The cytometric immunofluorescence technique used in this experiment has previously been shown to provide a useful assessment of deficiencies of mitochondrial protein expression (177) in terms of the individual subunits of the mitochondrial respiratory chain. As the subunit I of complex IV is encoded for by the mtDNA molecule, and this complex has previously been found to be deficient in muscle tissue (98) and central nervous tissue (178) of patients with ALS, the possible genetic origins of such a defect were investigated. Even without finding a discrete enzyme complex defect in homogenates of cultured myoblasts and fibroblasts, it should be possible to demonstrate abnormalities in the mitochondrial expression of individual cells, where perhaps these are being masked by a large population of phenotypically normal cells without any deficiencies of protein expression below the threshold of the expression of a mtDNA mutation.

The deletions and lower levels of mtDNA from patient muscle and spinal cord, as found in some studies (112,113,178), would suggest that these abnormalities produce a pathological effect through respiratory chain complex deficiencies (179), because these are partly encoded for by the mtDNA molecule (complexes I, II and IV). Although respiratory chain enzyme function abnormalities have been described in many tissues (see above), there have been no hard correlations between mtDNA abnormalities and aberrant mitochondrial function in ALS. This is supported by the results as detailed.

Apart from 2 reported patients, one with a mitochondria tRNA(Ile) (4274T→C) mutation(180), and one with a heteroplasmic 5-bp deletion in the 5' end of the COX I gene (99), specific mitochondrial mutations have yet to be found in patients with ALS. The finding of lower levels of mtDNA in patient skeletal muscle (112) or higher levels of deletions (113) in central nervous system tissues without specific nuclear gene abnormalities, implies that various abnormalities of the mtDNA may be the primary cause of cellular abnormalities in the disease. In this respect, examination of the expression of subunit I of complex IV is likely to be of as much value in the assessment of mitochondrial protein expression as any of the other mtDNA-encoded proteins. These abnormalities are not found in all patients, however. A correlation has never been made between severity of disease and the extent of the mitochondrial genetic abnormalities described (115). Because mtDNA is susceptible to oxidative damage (26) and because the abnormalities of motor neurons in ALS may represent the result of oxidative damage (26,181), it would be difficult to state with certainty that the mtDNA abnormalities seen in the disease are not the secondary effects of oxidative damage rather than the cause *per se*.

The results from this single cell analysis are consistent with the finding of normal mitochondrial function from the patient cell lines analysed. It would be interesting to know whether mtDNA protein expression abnormalities were detected in the samples previously described where discrete respiratory chain enzyme deficiencies appear to occur. MtDNA analysis of the samples studied is described in the following chapter (8).

8 Analysis of mtDNA from Controls and Patients with ALS

8.1 Aims

To analyse mtDNA fractions from patient and control tissue in ~~order~~ to determine whether there are different amounts or a greater frequency of deletions in patients with ALS compared with controls.

8.2 Introduction

Mitochondria have their own DNA molecules which contribute to the biosynthesis of four of the five OXPHOS complexes on the mitochondrial membrane (I, III, IV, V) (Figure 8.1).

It would be predicted that abnormalities of electron transport chain function or other mitochondrial processes may be due to an underlying abnormality of the mtDNA. As mtDNA is more sensitive to oxidative damage than nuclear DNA (25), a degenerative disease, such as ALS may be related to cumulative insults to the mtDNA which would then manifest as mitochondrial dysfunction, with resultant cell death. To this end, mtDNA was extracted and analysed from muscle biopsies of the patients and controls in our sample groups.

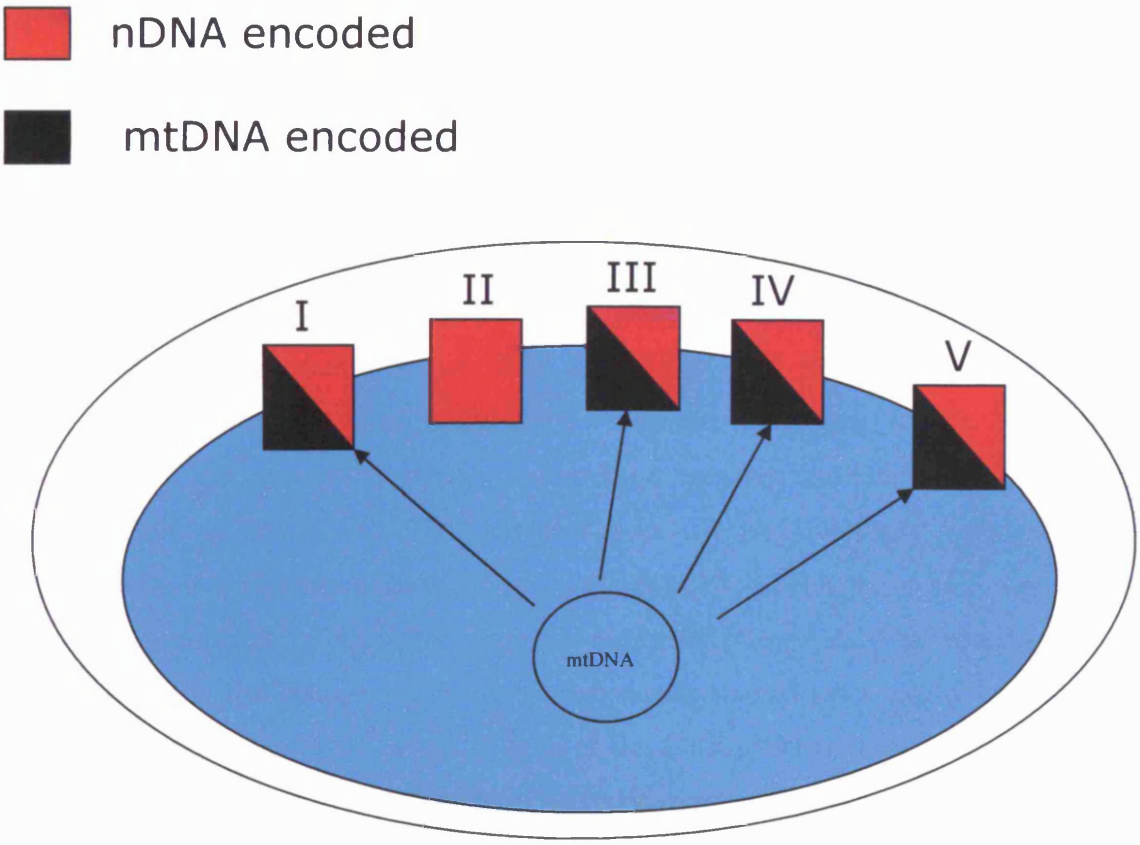


Figure 8.1 A schematic of a mitochondrion showing the mtDNA encoding the mitochondrial OXPHOS complexes on the inner membrane of the mitochondrion. Mitochondrial DNA encodes for subunits of four of the five complexes.

Mitochondrial DNA analysis has previously revealed that patients with ALS have a greater level of the so-called "common" deletion (4977 bp) in their central nervous system (113) and skeletal muscle (115) than controls. This finding was thought to support the role of mitochondria in the pathogenesis of ALS. mtDNA depletion has also been described in the skeletal muscle (112) of patients with ALS. Analysis of spinal cord (178) homogentate of patients with ALS revealed increased levels of mtDNA point mutations. Further evidence to support the idea of mtDNA abnormalities being involved in the pathogenesis of ALS was provided by the description of a patient with a motor neuron disorder and a specific deletion of the mtDNA gene encoding for subunit I of respiratory complex IV (99), although this patient had an earlier age of onset and predominantly

upper motor neuron involvement. Apart from this single case study, primary genetic causality of mtDNA abnormalities in ALS has not been convincingly demonstrated.

8.3 Approach

In the examination of mitochondrial abnormalities, total genomic DNA was extracted from whole muscle tissue (2.10.1) and subjected to restriction digest using *PvuII* enzyme in order to linearise the circular mtDNA molecules (2.10.2). Samples (3 μg) were loaded onto an agarose gel based on spectrophotometric calculations of concentration of the DNA. The samples were electrophoretically separated and transferred to a membrane to make a Southern blot (2.10.3). This initial blot was probed for mtDNA using a radiolabelled mtDNA fragment (Figure 8.2) to detect mtDNA rearrangements. The picture of the ethidium bromide-stained gel from this experiment was used to determine the optimum loading of the DNA samples to give equivalence of loading amounts. The amount of staining was measured using the ImageQuant software package (Amersham). Based on these calculations, a second Southern blot was prepared and hybridised simultaneously with a radiolabelled mtDNA fragment and a radiolabelled fragment of the nuclear 18S RNA gene. The latter probe was used to allow corrections to made for DNA loading.

The nuclear and mtDNA signals were measured by phophoimaging and analysed with ImageQuant software to determine the relative mtDNA levels in the muscle samples.

The tissue aconitase levels determined previously for whole muscle (chapter 4) were correlated with mtDNA levels to determine whether there was a relationship between oxidative damage and mtDNA levels *per se*.

In order to control for variability in the number of mitochondria, the absolute mtDNA : nDNA ratios were plotted against tissue citrate synthase concentrations (as previously determined – 2.8.6), as this is a marker for total tissue mitochondrial concentration.

To further investigate mtDNA rearrangements, the more sensitive long-range PCR (lrPCR) was used. In this procedure, deleted mtDNA molecules are preferentially amplified over full-length wild type species. The PCR was performed using with the Expand lrPCR kit (Roche), using the conditions and reagents described above (2.10.4). A 9900-bp region of mtDNA (6222 – 16152) was amplified. The muscle DNA samples used in the PCR amplifications were the same as the samples used for the Southern blots. As a preliminary experiment, samples of patient (PA11) and control (C5) DNA were loaded onto a gel in 5 lanes (a-e) with different primer and nucleotide concentrations (a=1×primer 1×dNTP, b=2×primer 1×dNTP, c=1×primer 2×dNTP, d=2×primer 2×dNTP, e=2×primer 1.5×dNTP) (figure 8.8).The test sample with optimum band visualisation was used to determine the primer and nucleotide concentrations which would be used in the lrPCR. In order to determine the levels of mtDNA deletions in the lrPCR experiment, the number of bands visible on an agarose gel, stained with ethidium bromide, was counted for each sample. The counting process was done in a "blinded" fashion, so that the experimenter did not know the identity of the samples for which the bands were being counted. This procedure has been performed in similar experiments using this method to look for deletions of the mtDNA (182).

8.4 Results

8.4.1 Primary Southern Blot

The initial blot probed with a radiolabelled mtDNA fragment did not demonstrate large scale mtDNA rearrangements.(figure 8.2). The levels shown were used to determine optimal loading amounts for equivalence on a second sample.

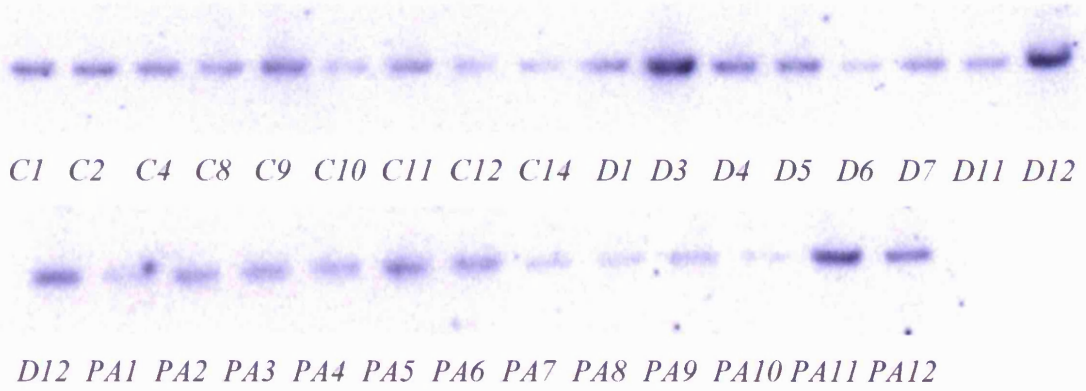


Figure 8.2 Primary Southern blot probed with radiolabelled mtDNA to reveal possible mtDNA rearrangements.

8.4.2 MtDNA quantification

The amount of mtDNA as determined by the mtDNA : n DNA ratio was the same in the patient (n=12), control (n=14) and disease control (n=4) muscle DNA samples as measured in the Southern blots (Figure 8.3, table 8.1).

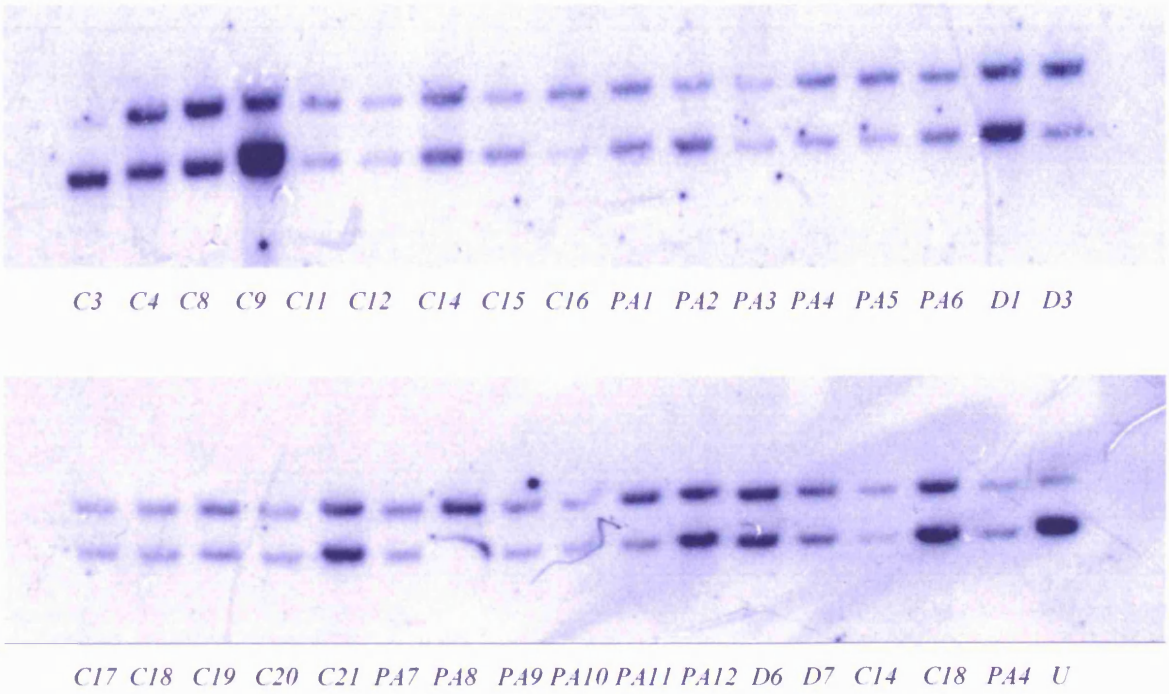


Figure 8.3 Southern blots comparing amounts of mitochondrial (upper band) and nuclear (lower band) DNA. U is sample from a known paediatric patient with mtDNA depletion. The other samples are labelled according to their allocated experimental code (table 2.1-2.3). Sample PA8 has an air bubble on the lower band. This sample was, therefore, not used for analysis.

Table 8.1 mtDNA:nuclear DNA ratios for muscle DNA

Sample Type	Number of Samples	Mean mtDNA : nuclear DNA ratio
Patient	11*	1.09 ± 0.407
Control	12	0.98 ± 0.398
Disease Control	4	1.02 ± 0.096

* Sample PA8 was not used due to the artifact described above (figure 8.4)

No significant correlation was demonstrated between mtDNA concentration and citrate synthase concentration (figure 8.4), indicating, that variations in the levels of mtDNA observed are not due to variations in absolute mitochondrial number.

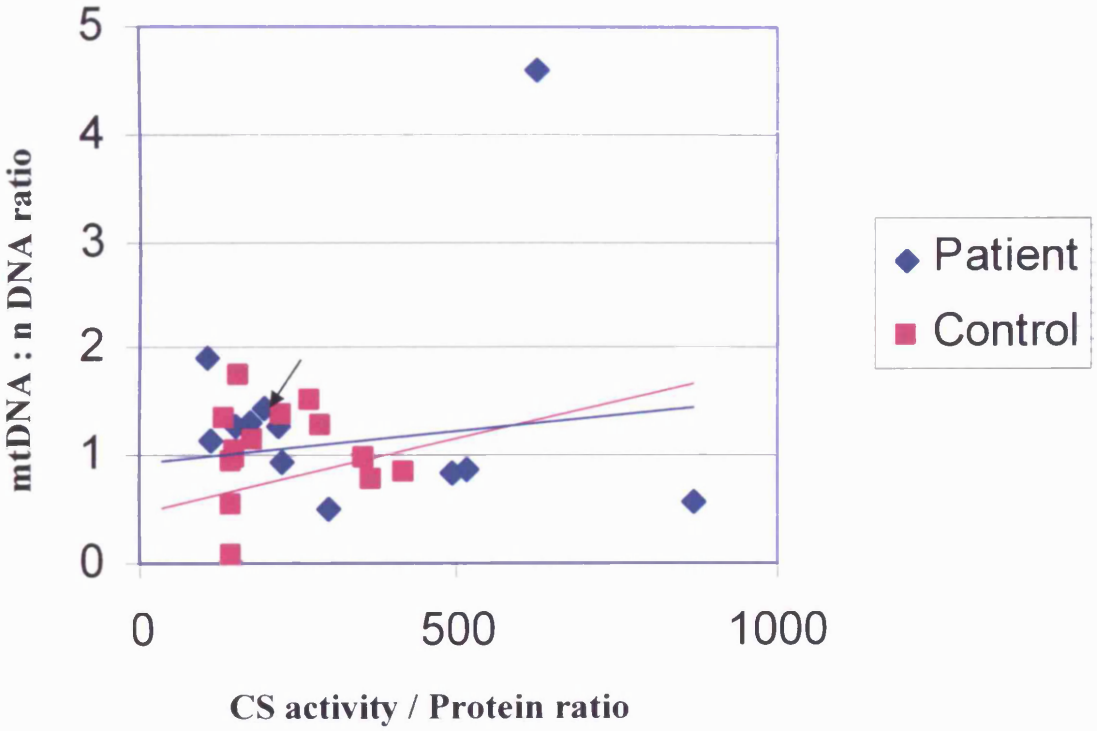


Figure 8.4 mtDNA:nuclear DNA ratios against tissue citrate synthase activity (nmoles.min^{-1}.mg^{-1}) demonstrating no difference in amount of mtDNA per mitochondrial volume between patient and control samples. Disease and normal controls have been pooled. The SOD1 mutant sample (PA11) has been arrowed.

8.4.3 MtDNA levels and tissue aconitase activity

There was a negative correlation between the levels of muscle aconitase determined by spectrophotometric analysis (chapter 5) and relative mtDNA levels determined by Southern blotting (Figure 8.5), although this did not reach significance ($p=0.093$).

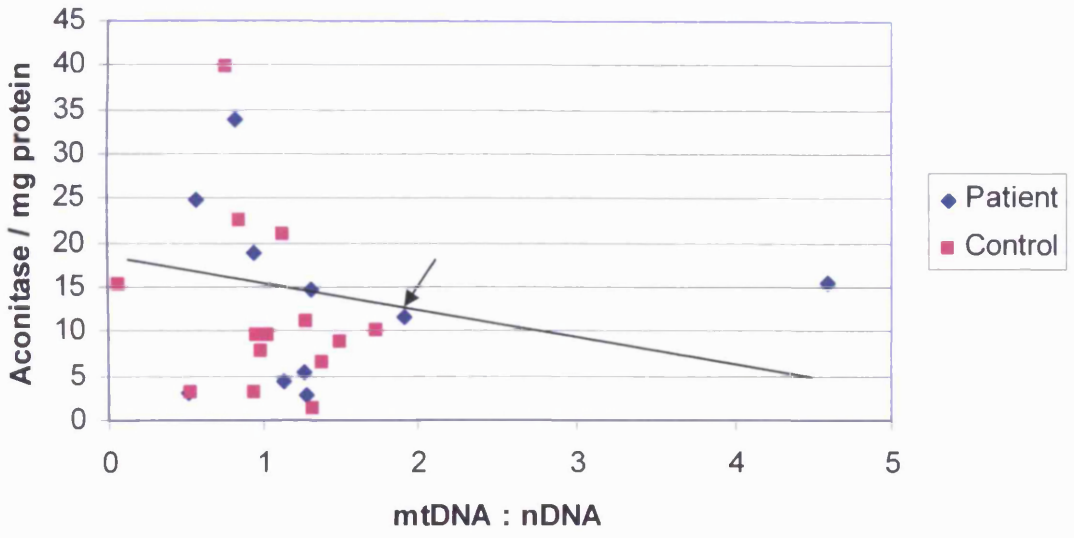


Figure 8.5 Correlation of tissue aconitase activity (nmoles.min^{-1}.mg^{-1}) and mtDNA levels in patient and control muscle samples. Disease and normal controls have been pooled. The SOD1 mutant sample (PA11) has been arrowed.

In order verify whether this relationship was true for other tissues, fibroblasts with a known single, large mitochondrial deletion, partial depletion (residual levels of mtDNA of 25%) and normal levels of mtDNA (provided courtesy of Dr J-W Taanman) were grown up and subjected to the same analyses. There was a difference in aconitase levels between theses three samples (table 8.2), but in contrast to the results for muscle, which suggested that low mtDNA levels are associated with relatively high aconitase activity levels, the mtDNA-depleted fibroblasts showed a lower Aconitase activity the the two control fibroblast samples.

Table 8.2 Aconitase activity in fibroblasts with different levels of mtDNA

Type of mtDNA abnormality	Aconitase activity (U/mg)
None (control)	3.45
Deletion	2.32
Partial depletion	1.55
None (control)	2.49

8.4.4 Age and mtDNA levels in muscle tissue

There was no correlation between patient or control subject age and the absolute amount of mtDNA present (figure. 8.6).

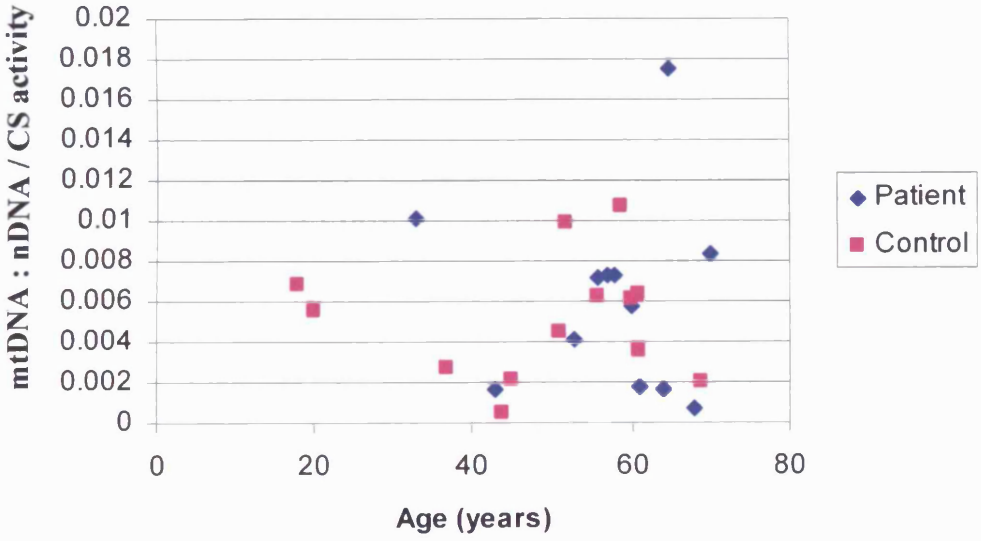


Figure 8.6 The relation between the amount of mtDNA present in muscle tissue and subject age. The amount of mtDNA is given relative to citrate synthase (CS) activity to correct for a possible difference in amount of mitochondria present in the sample.

8.4.5 Determination of Reagent Concentration for lrPCR

The Optimum concentration of primers and nucleotides required to visualise multiple bands using lrPCR were determined by the best visualised bands using the concentration of primers and nucleotides as shown (figure 8.7)

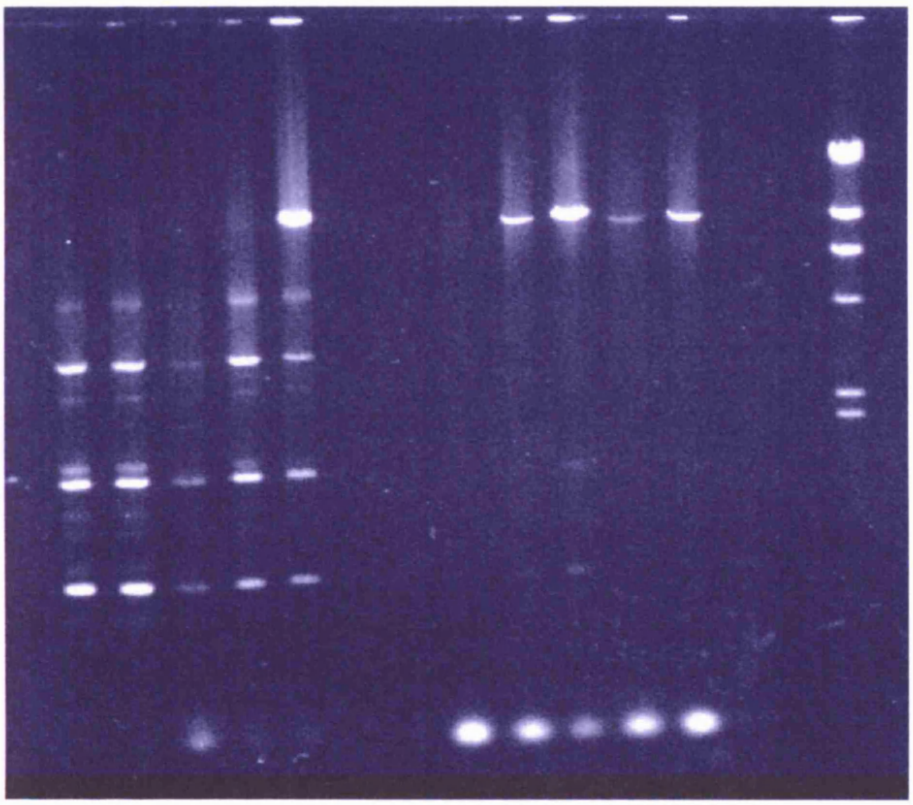


Figure 8.7 Agarose gel of lrPCR products obtained using variable nucleotide and primer concentrations in order to determine the optimum concentrations for visualization of multiple bands.

The optimum absolute amounts of reagents for the lrPCR were determined to be;

DNA -	200 ng
dNTP (200 mM)-	350 µl
Forward primer-	300 nm
Reverse primer-	300 nm

8.4.6 Band quantification using lrPCR

Long-range PCR of the mtDNA revealed multiple deletions, as indicated by the appearance of multiple bands in some of the patient samples as well as some of the control samples (figure 8.8). Long range PCR is able to detect low levels of mtDNA deletions that are not identified on by Southern blotting. The "normal" band of amplified mtDNA, without deletions, is visualised at around 8kb. Twelve patient samples and sixteen normal- and disease-controls were used. There was no increase in the number of deletions present in patient samples as compared with controls (table 8.3).

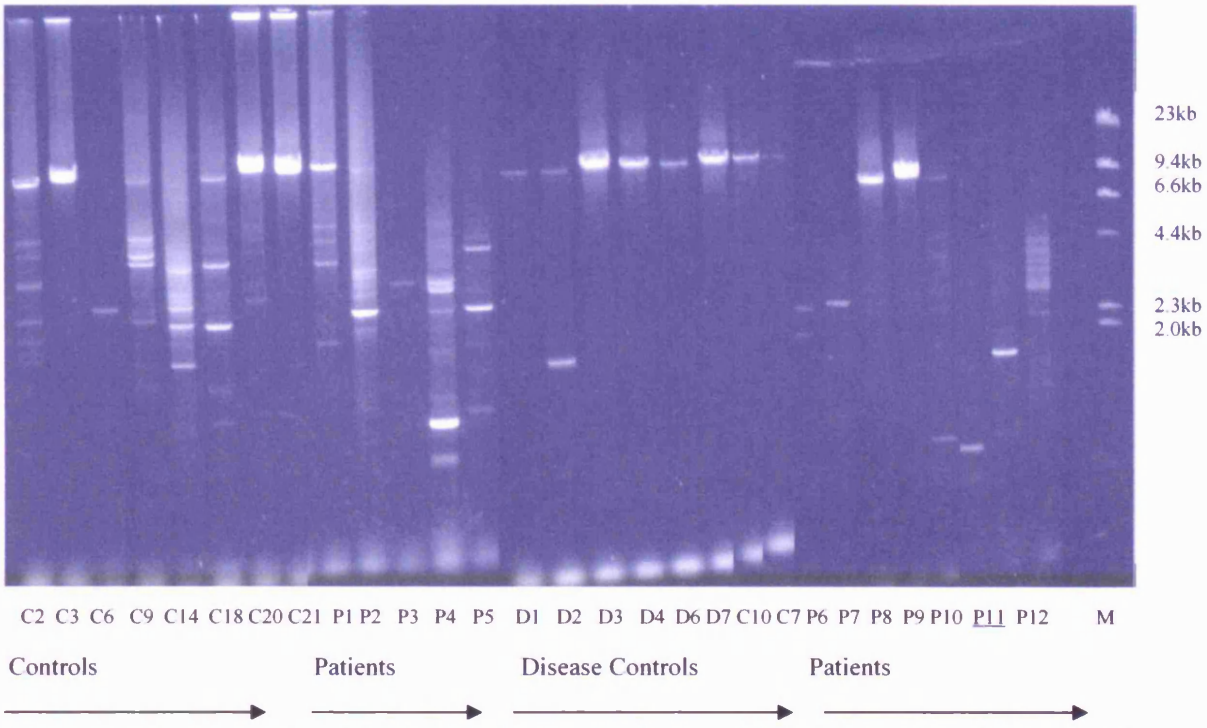


Figure 8.8 Agarose gels of lrPCR products stained with ethidium bromide, showing total number of bands of PCR-amplified mtDNA. C=normal control P=patient D=disease control M=bacteriophage λ DNA digested with HindIII (DNA standard). The SOD1 mutant sample (PA11) is underlined.

Table 8.3 The mean number of bands seen on lrPCR analysis of mtDNA extracted from skeletal muscle

Sample Type	Number of Samples	Mean Number of Bands
Control	12	3.75
Disease Control	8	1.63
Patients	10	3.4

For patient, normal- and disease control mtDNA lrPCR here was a positive association between subject age and the number of bands present, although this did not reach statistical significance. (p=0.24 for all samples) (figure. 8.9).

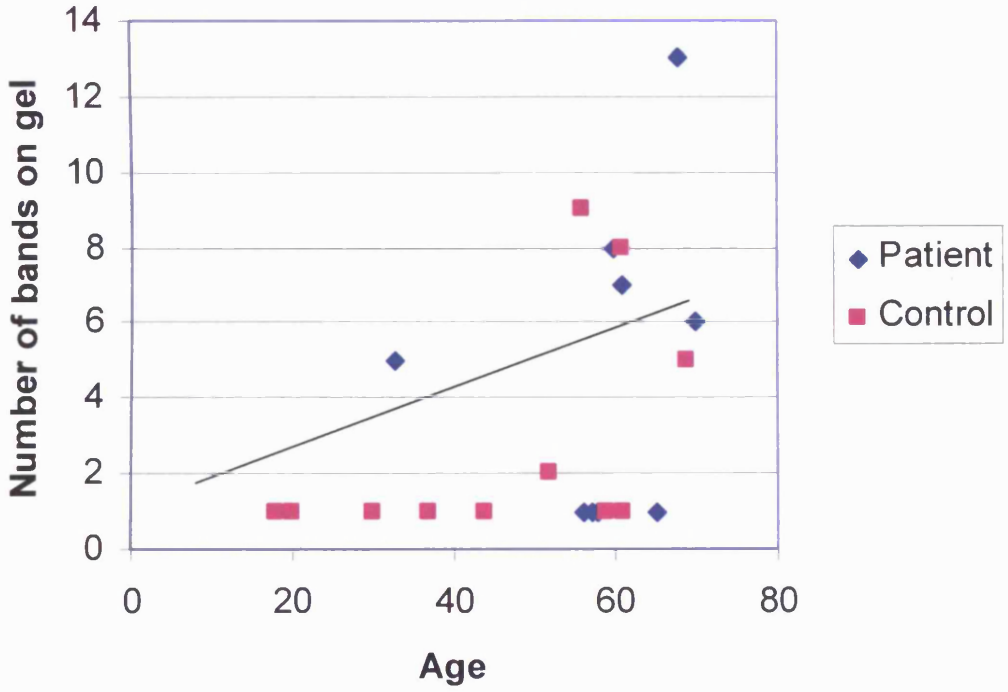


Figure 8.9 Association of age and number of bands visualised on lrPCR using pooled patient and control samples

There was a negative association between the number of bands present on lrPCR and amount of mtDNA as measured by Southern blotting for each sample (p=.095) (figure. 8.10). This finding may represent a reduction in the total amount of mtDNA present in the muscle as a cumulative effect of the multiple deletions present.

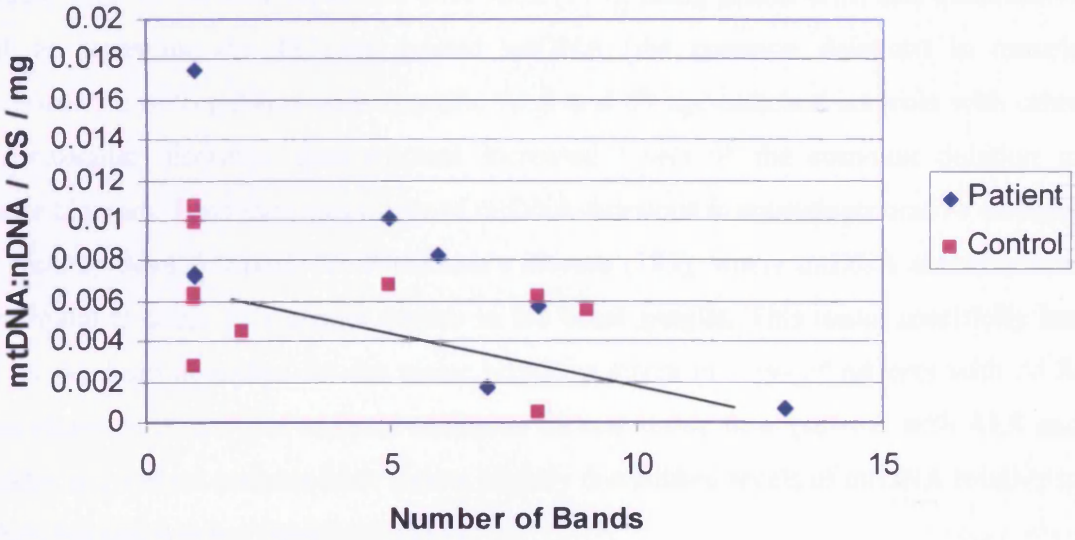


Figure 8.10 Relationship between number of bands seen on lrPCR and relative amount of mtDNA per tissue citrate synthase (CS) activity per mg of protein.

8.5 Discussion

Researchers have previously identified lower levels of mtDNA in the skeletal muscle of 13 patients with ALS as compared with 17 controls using identical Southern blotting techniques (112). This study was performed using patients with spinal muscular atrophy as controls, and the differences in absolute mtDNA levels did not correct when controlled for citrate synthase concentration, indicating that differences were not due to differences in mitochondrial concentration *per se*. This finding of lower levels of mtDNA does not appear to correlate with increased levels of mtDNA deletions. In a study looking at levels of the 4977-kb common deletion of mtDNA in single cell post mortem brain and spinal cord samples (116) spinal cord neurons showed slightly higher deletion detection

rate in patients than controls (94% vs 75%). No significant differences were found between patients and controls for neurons derived from other motor or non-motor regions. The analysis of the mtDNA from the sites of "pathology" would suggest that mitochondrial deletions are not a significant contributory factor, and one would not expect to see large scale abnormalities in skeletal muscle on the basis of this result. A separate study of hospital inpatients with ALS (115), using primer-shift and quantitative PCR to determine the 4977-bp deleted mtDNA (the common deletion) in muscle specimens from 36 patients with sporadic ALS and 69 age-matched controls with other neuromuscular disorders, demonstrated increased levels of the common deletion in muscle biopsies. The tissue specificity of mtDNA deletions in neurodegenerative diseases has recently been described for Parkinson's disease (183), where mtDNA abnormalities were found to occur to a greater degree in the basal ganglia. This tissue specificity has not, as yet, been described for the motor cortex or motor neurons of patients with ALS. More recent Southern blot analysis studies of muscle tissue from patients with ALS and disease- and normal controls have shown slightly diminished levels of mtDNA relative to nDNA, but this was not significant (117).

Are measurements of the common deletion or of total mtDNA concentrations appropriate measures to look for mtDNA abnormalities in ALS? A patient with known autosomal dominant progressive external ophthalmoplegia was shown to have normal levels of mtDNA on Southern blotting, but multiple deletions on long-range PCR (184). The techniques available may not be specific or sensitive enough to detect very low levels of mtDNA deletions. It would appear from the assessment of mitochondrial protein expression (chapter 7), that there is no evidence of mtDNA abnormalities producing a deficiency in subunit 1 of complex IV expression in any of the thousands of cultured cells studied. This would lend support to the finding of normal levels of mtDNA in our patient and control muscle tissue samples, unless the mitochondrial damage was occuring at insufficient levels to result in abnormalities of protein expression in any of the cells studied. The finding of normal mtDNA levels is also consistent with the observation of normal respiratory chain activities in the tissue and cultured cell samples used in this study. Whether or not it is valid to make statements regarding mtDNA in muscle tissue

on the basis of experiments conducted on cultured fibroblasts, there is a consistency in the pattern of results between tissues and cells studied.

It is interesting to note that in our sample group, increasing age did correlate with an increased level of mtDNA deletions as determined by lrPCR analysis, a feature which has also been described for normal ageing (114,159,185). The accumulation of mtDNA deletions during ageing may be the result of oxidative damage sustained by the ageing mtDNA molecules. The fact that the absolute levels of mtDNA, as determined by Southern blotting, were not found to be diminished in association with aging in our sample, is consistent with published data (42). Increasing numbers of mtDNA deletions were statistically associated with lower total levels of mtDNA (8.10) which is, perhaps, surprising, given the small overall amounts of mtDNA lost in deletions.

There was a negative correlation between tissue aconitase levels and tissue mtDNA levels. This may imply that oxidative damage may not have a deleterious effect on mtDNA concentrations, although this does not appear to be a feature peculiar to ALS, being seen in all of the samples studied. The fibroblast cultures known to have mtDNA abnormalities (table 8.2) did not show any consistent relationship between mtDNA levels and cellular aconitase activity.

9 Cellular Response to Oxidative Stress

9.1 Aims

To examine the apoptosis and oxidative damage profiles of patient and control cell cultures in response to exogenous oxidative stress.

9.2 Introduction

Cell dysfunction and death in response to oxidative stress is a well described model of neurodegenerative disease (186). It is thought that the generation of free radicals and their subsequent mishandling result in the death of neurons and the development of disease through the accumulation of oxidatively modified protein. As far as ALS is concerned, the discovery of mutated forms of SOD1, an antioxidant, in a sub-group of familial cases has led to an increasing focus on the role of oxidative damage in the pathogenesis of the disease. Although markers of oxidative damage have been shown to be elevated in the central nervous system tissue of patients with ALS (89), the time course and role in the aetiology of the disease has not been clearly delineated (181).

Increased vulnerability to injury of motor neurons in ALS has been described in the G93A transgenic SOD1 mouse model (187). In this study, injury to the sciatic nerve in SOD1(G93A) mice resulted in an acceleration in disease progression so that 90 day old mice show deficits that are only seen at the end stage in uninjured SOD1(G93A)mice.

There is, however, no reason why the levels of oxidative stress to which patient nervous system tissues are subjected would be different to those of controls, and no clear relationship has been demonstrated between external factors and the aetiology of ALS (188).

Oxidative damage is not a phenomenon confined to the central nervous system of patients with ALS. Evidence of increased damage to muscle tissue of patients with ALS

(depleted levels of ATP and elevated levels of mitochondrial uncoupling protein) has also been found, which is felt to be the result of specific metabolic impairments in the tissues studied (148).

In view of the mitochondrial focus of the study, paraquat was chosen as a model of oxidative stress, as this is known to specifically affect mitochondrial respiratory function by the production of superoxide anions generated from paraquat radicals and by singlet oxygen derived from anions (189). This was felt to be a more quantifiable and reproducible model of oxidative stress than, for example, serum withdrawal from the growth media, which other researchers have used (80).

While there is an accumulating body of evidence that oxidative damage is involved in the pathogenesis of ALS, it is not clear by what mechanism this damage is having a deleterious effect on the tissues affected. In order to investigate the mechanisms by which cell death may occur, separate analyses were performed for apoptosis (caspase activation) and the total levels of oxidative damage sustained by the tissue (aconitase activity). These experiments were conducted on cybrids, myoblasts and fibroblasts.

Aconitase activity is a marker of oxidative damage because the enzyme is rendered inactive through oxidation of an iron-sulphur cluster $(4FeS)^{2+} \rightarrow (3FeS)^{+}$. The enzyme is predominantly located intramitochondrially, although some aconitase is present in the cytosol. Glutamate has been shown to inhibit aconitase activity in rat neurones at low levels (190). Aconitase activity is also very sensitive to inhibition by peroxynitate, but not by its precursor, nitric oxide (191).

Work has been carried out looking at the mechanisms of cell death in ALS. Much of this has focussed on neuronal cell cultures (192) and cell models over-expressing SOD1 (193). In ALS, dying neurons undergo programmed cell death (PCD), rather than necrosis. Post mortem specimens and cell cultures exhibit features reminiscent of apoptosis, a prominent morphological form of PCD. In addition, many key molecular components of the PCD machinery are activated in ALS spinal cords (194). In order to analyse mechanisms of cell damage in response to oxidative stress, caspase (apoptosis) assays were performed on cultures treated with paraquat.

9.3 Approach

Cell cultures of myoblasts, fibroblasts and cybrids were grown on cell culture plates as described (2.5). All cell cultures were examined at the same passage number and using the same experimental conditions. A stock solution of 100 mM paraquat in H_2O was added to the cell culture medium of the growing cells to make concentrations of 0.1 mM and 1 mM. Aconitase activity was measured 24 h later. These paraquat concentrations had been derived by assessing the response of a number of cell cultures to increasing paraquat concentrations. Increasing the concentration of the paraquat much above 1 mM resulted in almost complete abolition of aconitase activity, while obvious effects on cell death were seen at concentrations as low as 0.1 mM, it was considered that growing cultures in normal media and these two paraquat concentrations would provide useful experimental comparison between patient and control cell samples. Further analysis was conducted on myoblast cultures with paraquat concentrations of 0.05 mM, 0.1 mM and 1 mM. Aconitase activities were corrected for protein concentration of the cell homogenate under analysis.

In situ caspase 3 activity staining was used to measure the degree of apoptosis undergone by the cell cultures and was assessed using a fluorescent marker of activity (2.11.1) (figure 9.1). The cell cultures were grown in culture medium supplemented with the appropriate concentrations of paraquat as described, and the activity assayed 24 h later.

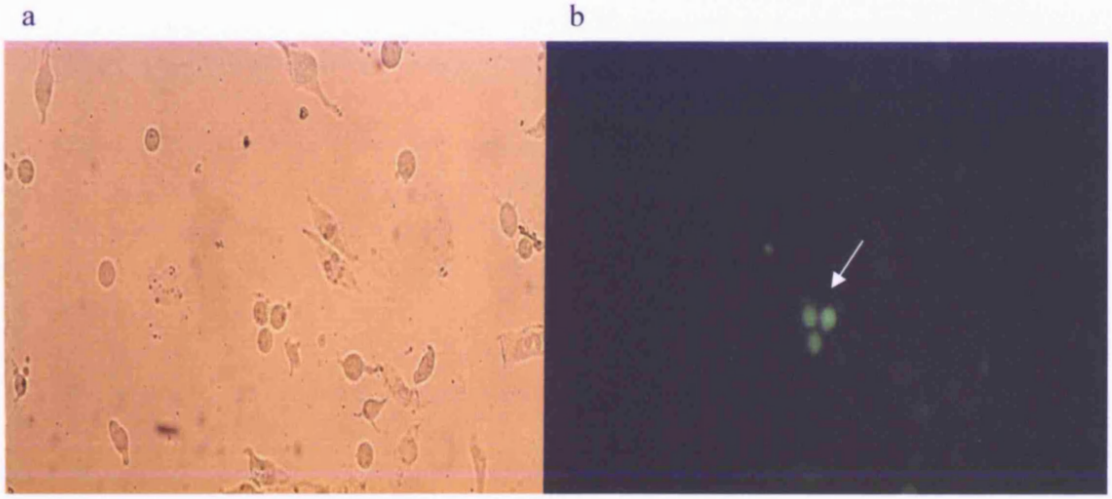


Figure 9.1 *Cultured fibroblasts under phase contrast (a) and viewed with a fluorescent microscope (b). Three cells out of the total field demonstrate caspase 3 activation (arrowed).*

9.4 Results

9.4.1 Oxidative Stress

There was a significantly greater fall in aconitase activity in patient myoblasts ($p=0.04$) and fibroblasts ($p=0.02$) cultured at a paraquat concentration of 0.1mM than controls (figure 9.2 – 9.4, table 9.1). This difference was not observed in platelet-derived cybrids or in myoblasts grown at concentrations of 0.05 mM or 1 mM paraquat nor in fibroblasts grown in 1 mM paraquat. There is a relatively broad spread of results seen for all aconitase activity assays (figure 9.2 – 9.4)

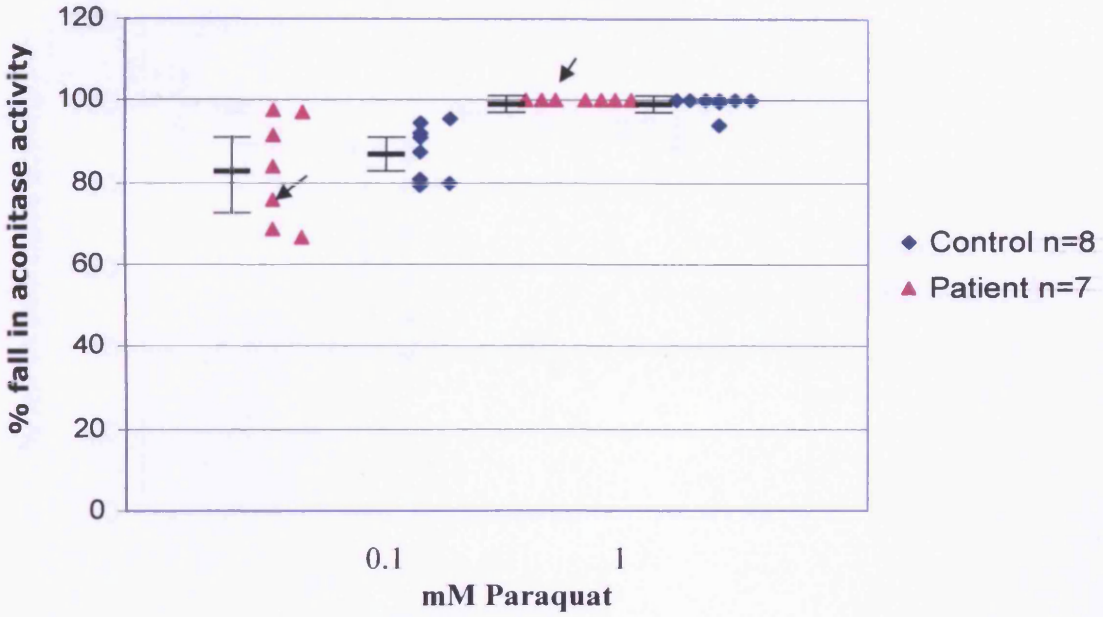


Figure 9.2 Percentage fall in aconitase activity of cybrid cell cultures grown in different concentrations of paraquat showing spread of values, mean and SD. The values for the mutant SOD1 sample (PA11) are arrowed.

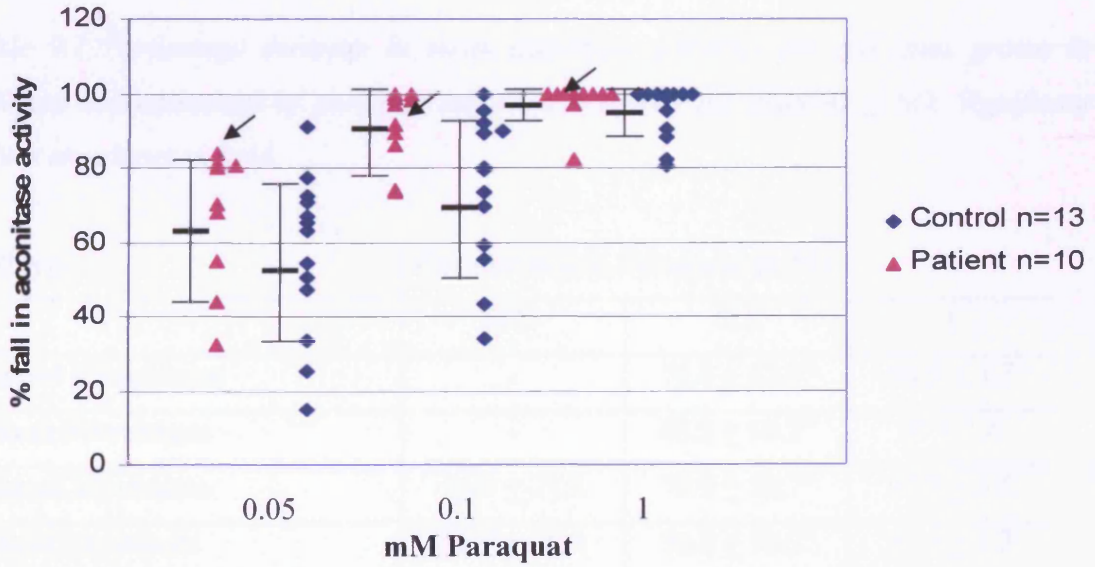


Figure 9.3 Percentage fall in aconitase activity of myoblast cell cultures grown in different concentrations of paraquat showing spread of values, mean and SD. The values for the mutant SOD1 sample (PA11) are arrowed.

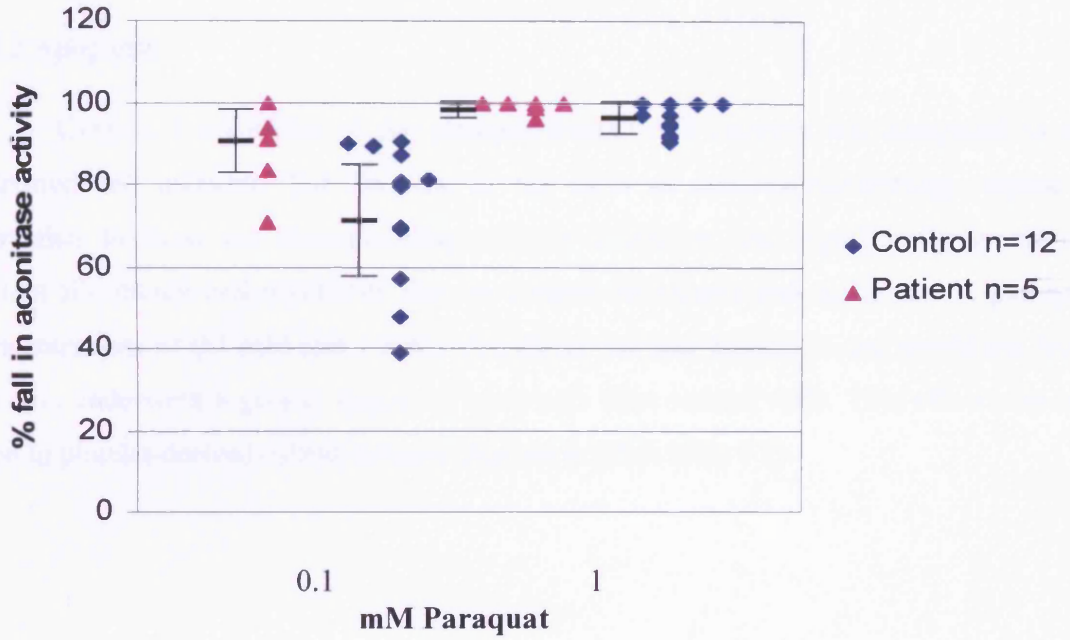


Figure 9.4 Percentage fall in aconitase activity of fibroblast cell cultures grown in different concentrations of paraquat showing spread of values, mean and SD. The SOD1 mutant sample was not available for analysis.

Table 9.1 Percentage decrease in mean aconitase activities for cell lines grown in different concentrations of paraquat (absolute activity / mg protein) ± SD. Significant values are shown in bold.

Cell type	**Concentration Paraquat (mM)**		
	0.05	0.1	1
Control Fibroblasts	-	**73.4 ± 17.0[b]**	96.5 ± 3.7
Patient Fibroblasts	-	**88.3 ± 10.2[b]**	99 ± 1.6
Control Myoblasts	56.3 ± 21.8	**73.9 ± 20.7[a]**	97.9 ± 5.6
Patient Myoblasts	62.7 ± 20.4	**90.8 ± 10.2[a]**	95.1 ± 7.2
Control Cybrids	-	87.5 ± 6.7	99.2 ± 2.1
Patient Cybrids	-	83.0 ± 13	100.0 ± 0

[a]Significant difference (p=0.04) [b]Significant difference (p=0.02)

9.4.2 Apoptosis

Caspase 3 activation of the paraquat-treated cell cultures was compared to the untreated cell cultures. The increase in the ratio of cells demonstrating caspase 3 activation to those not demonstrating caspase activation was significantly greater in patient fibroblasts and myoblasts than in control fibroblasts and myoblasts at paraquat concentrations of 0.1 mM and 1 mM. Therefore, cultured fibroblast and myoblasts from patients underwent a greater degree of apoptosis than control cells. This effect was not seen in platelet-derived cybrid cultures (figures 9.5-9.8, table 9.2).

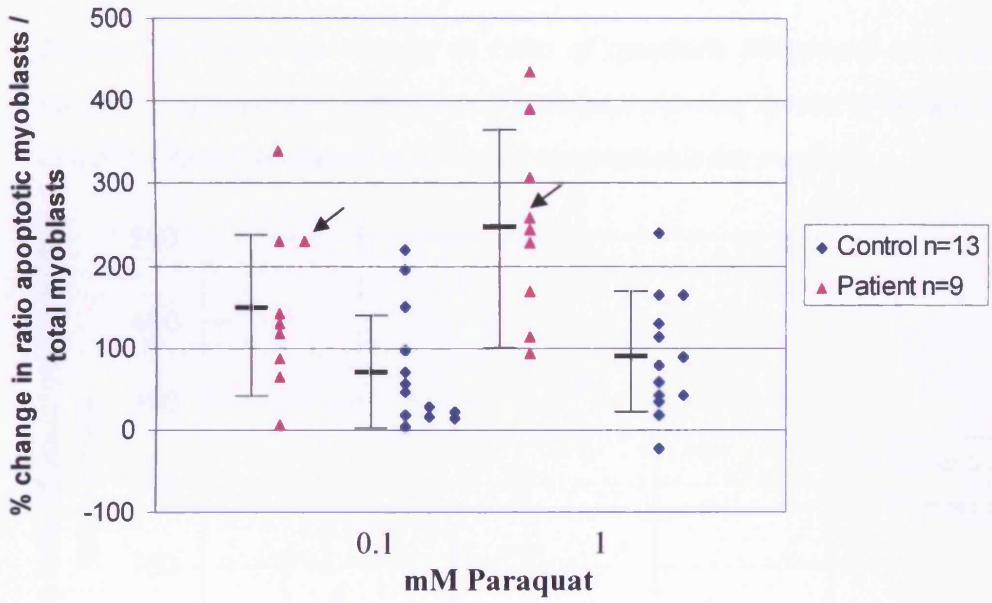


Figure 9.5 Percentage change in ratio of apoptotic myoblasts to total cell number at different concentrations of paraquat showing spread of values, mean and SD. Values for the mutant SOD1 sample (PA11) are arrowed.

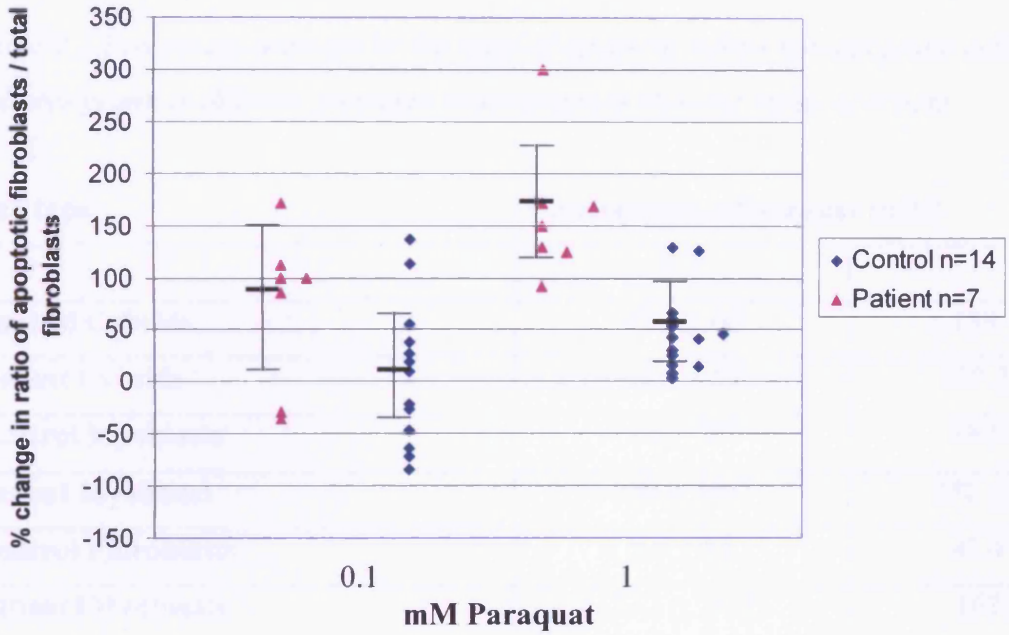


Figure 9.6 Percentage change in ratio of apoptotic fibroblasts to total cell number at different concentrations of paraquat showing spread of values, mean and SD. The SOD1 mutant sample was not available for analysis.

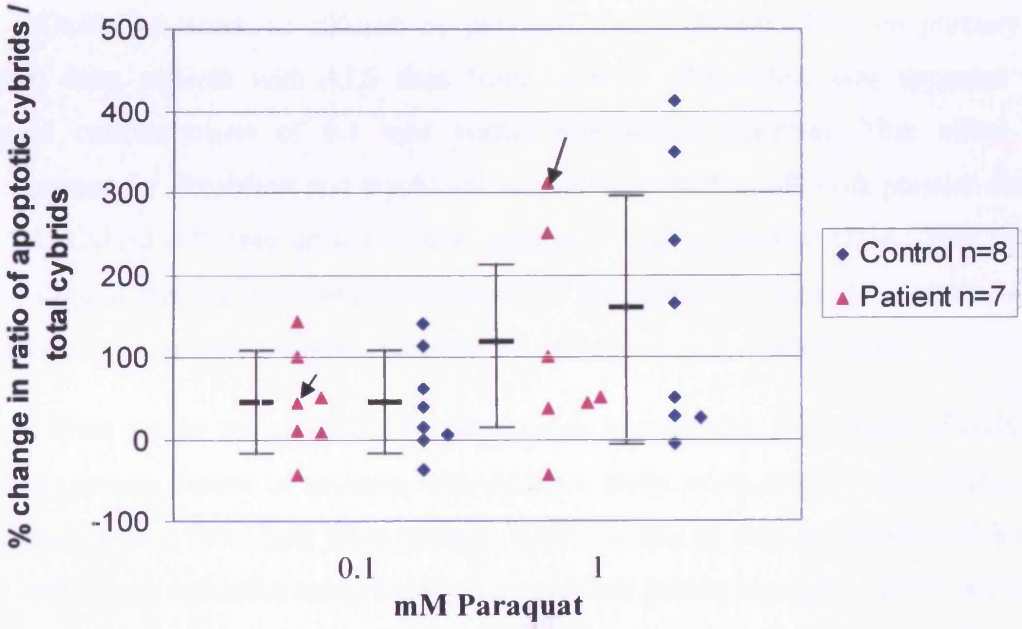


Figure 9.7 Percentage change in ratio of apoptotic cybrids to total cell number at different concentrations of paraquat showing spread of values, mean and SD. Values for the mutant SOD1 sample (PA11) are shown (arrowed).

Table 9.2 Percentage decrease in the ratio of apoptotic cells / non-apoptotic cells of cell cultures grown in different paraquat concentrations showing range of results

Cell type	Concentration Paraquat (mM)	
	0.1	1
Control Cybrids	42.2 ± 60	158 ± 160
Patient Cybrids	44.5 ± 62	107.2 ± 127
Control Myoblasts	**72 ± 73[a]**	**88.5 ± 72[b]**
Patient Myoblasts	**150 ± 101[a]**	**250 ± 115.8[b]**
Control Fibroblasts	5.4 ± 64	**47.4 ± 39[c]**
Patient Fibroblasts	72.1 ± 76.5	**162 ± 67[c]**

[a]Significant difference (p=0.048) [b]Significant difference (p=0.003) [c]Significant difference (p=0.003).

9.5 Discussion

Oxidative stress, as induced by paraquat, had a greater effect on primary cell cultures from patients with ALS than from controls. This effect was apparent with paraquat concentrations of 0.1 mM within the growth medium. This effect was demonstrated for fibroblasts and myoblasts but not for cybrids made with platelet-derived mtDNA. Cybrid cell lines do not contain patient or control nuclear DNA. This finding would suggest that the increased vulnerability of the patient cell lines to oxidative stress is mediated by a nuclear-encoded rather than a mitochondrial-encoded factor.

These results are supported by other work investigating the effects of oxidative stress on remote tissues of patients with ALS. A study using cultured fibroblasts (56) demonstrated that fibroblasts from patients with sporadic as well as familial ALS were more sensitive to oxidative stress from the free radical generating agent, SIN-1 but not to serum withdrawal. Interestingly, addition of hydrogen peroxide induced greater levels of oxidative damage in the fibroblasts from familial patients with known SOD1 mutations than sporadic patients (56).

A neuronal cell model transfected with mutant SOD1 (79) showed increased cell death when exposed to oxidative stress by serum withdrawal, whereas the presence of normal human SOD1 exerted a protective effect. Under basal, unstressed culture conditions, no change in the ATP : ADP ratio was observed in the presence of mutant SOD1. These results support the assertion that neuronal damage is induced through increased sensitivity to oxidative stress. Serum withdrawal, which will affect the growth and metabolism of cells, is a relatively non-specific condition by which to induce oxidative stress, especially in view of the different types of sera and growth media used by different researchers. Measurement of glutamate uptake in response to oxidative stress using paraquat with the SOD1 G93A neuronal cell model revealed increased sensitivity to the paraquat in transfected cells relative to control cultures (82).

The increased levels of oxidative damage occurring in SOD1 mouse model motor neurons have been attenuated by researchers using antioxidants (195), such as vitamin E and creatine. As has been stated earlier, antioxidants appear to have little effect on disease progression or severity in patients with the ALS (196,197), apart from a single unblinded, self report study demonstrating a lower risk of dying of ALS in individuals using regular vitamin E supplements (93).

The finding of an increased level of sensitivity to damage from oxidative stress in remote tissues, such as cultured fibroblasts and myoblasts is clearly of questionable relevance to the function and activity of motor neurons *in situ*. However, as mutant SOD1 is expressed in every cell in the body, it would seem reasonable to extrapolate the influence of genetic factors to every cell where appropriate conditions have been met. Due to the obvious difficulties in the collection and analysis of such tissue, similar experiments in living motor neurons from affected patients would not be possible. Therefore, the finding of increased sensitivity to oxidative damage in remote tissues from patients with ALS is important in the consideration of the pathogenesis of the sporadic forms of the disease, where other cell models and transgenic mice are not available as experimental models.

The finding of an association between increased lactate production and free radical formation in the serum of ALS patients undergoing exercise (148) supports the

concept of an enhanced systemic sensitivity to oxidative stress. The lack of systemic abnormalities in the vast majority of familial ALS patients suggests that the motor neurons are a vulnerable site of free radical damage resulting from their particular metabolic requirements.

Caspase activation has been reported in the spinal cords of transgenic mouse models of ALS (198). Caspase 3, 9 and 12 activation was studied and nitrotyrosine staining was utilised, as a marker of oxidative stress. There was an increased rate of caspase cleavage, indicative of apoptotic pathway activation, and levels of oxidative stress were found to be higher in the same tissues. These concur with the increase in markers of oxidative stress and apoptosis found in remote cell cultures of patients with ALS, implying that a common molecular mechanism may be responsible. Increased levels of caspase activation, and hence apoptosis, are found in cell cultures grown in the same concentration of paraquat which is shown to induce oxidative stress. Therefore, oxidative stress is a possible cause of apoptosis in these cultures, which would support the suggestion that increased sensitivity to oxidative stress is the cause of motor neuron death through apoptosis in ALS.

10 Patterns of Inheritance in Familial ALS

10.1 Aims

To analyse patterns of inheritance in a large group of families with familial ALS. To determine whether the phenomenon of anticipation is demonstrated in inheritance. To determine whether there is a maternal pattern of inheritance in familial ALS.

10.2 Introduction

Sporadic and familial forms of ALS exist. The pattern of inheritance in familial ALS is usually autosomal dominant with age dependent penetrance (199). Mutations of the SOD1 gene, which encodes the enzyme copper/zinc superoxide dismutase, are found in approximately 20% of familial patients and occasional, apparently sporadic, patients (47). SOD1 mutations account for 1-2% of all patients with ALS (ALS1) (48). Other, rarer, genetic abnormalities have been detected in patients with ALS, including the alsin (53) and APEX (54) genes along with a number of other mutations (see table 1.1), but the significance of these is uncertain at the present time. The molecular and environmental causes of ALS otherwise remain undetermined.

Anticipation is the tendency for a disease to demonstrate an earlier age of onset or increasing severity of symptoms in successive generations. The concept was recognised over a century ago (200,201), and the term 'anticipation' was applied to observations of mental illness by Mott early in 1911 (202). A paper on myotonic dystrophy by Penrose in 1948 (203) suggested that the phenomenon was caused by several forms of bias. The discovery of a genetic basis for anticipation in some diseases was made in the early 1990s. Trinucleotide repeat expansions were found to be the cause of several diseases with genetic anticipation (204).

Anticipation has been investigated in a range of diseases. Psychiatric illness has received particular attention with anticipation demonstrated in schizophrenia (205-207),

panic disorder (208) and bipolar affective disorder (209). Other diseases have included inflammatory bowel disease (210-212), haematological malignancies (213-216), rheumatoid arthritis (217), familial cavernous angiomas (218) and polycystic kidney disease (219,220). Anticipation has been reported in a single Japanese family with the G93S mutation of SOD1 (221), as well as some Italian and American families with mutations at codon 84 of SOD1 (222).

ALS is a neurodegenerative disease, with some clinical and pathological similarities to conditions with demonstrated trinucleotide repeats, for example bulbospinal muscular atrophy, where the repeat is in the androgen receptor gene on the X chromosome (223). Functional and structural mitochondrial abnormalities have been demonstrated in different tissues of patients with ALS (164). Some researchers have identified mutations of the mtDNA in these tissues (112). The implication of mitochondrial genetic abnormalities in the pathogenesis of ALS suggests that these abnormalities may be reflected in a different mode of inheritance in SOD1 negative familial ALS. MtDNA disorders are maternally inherited (224) and one would, therefore, expect a sex effect in inheritance for SOD1 negative transmissions, if mtDNA mutations were causative.

Using the database of families with more than one individual affected by ALS, with the assistance of Professor deBelleroche from Imperial College London and Dr Marcus Bradley, the patterns of inheritance and ages of patients were examined to determine whether the phenomenon of anticipation occurs in families with ALS. Subgroup analysis was performed on known SOD1 negative and known SOD1 positive patients. The effect of sex on inheritance was analysed for SOD1 positive and SOD1 negative transmissions, to determine if maternal inheritance occurred significantly more often than paternal.

10.3 Approach

A total number of 185 families with two or more individuals affected by ALS were studied. These families are part of an ongoing study into the clinical and molecular genetics of ALS (199). Pedigree structure, age of onset of symptoms, duration of illness, and age of death of each individual was recorded. The superoxide dismutase-1 (SOD1) gene mutation status was recorded for each family.

Individual transmissions between generations were identified. Intergenerational values were compared between generation 1 (G1) and generation 2 (G2) for each individual transmission. G1 comprised fathers, mothers, uncles and aunts. G2 comprised sons, daughters, nephews and nieces. The differences in age of onset, duration of disease and age at death between G1 and G2 were then calculated.

For first degree transmissions, the number of cases where maternal inheritance occurred was compared with those where paternal inheritance took place. The frequencies were analysed for the SOD1 positive and negative families.

Initially all subgroups were analysed together. To minimise bias, direct transmissions were those between first degree relatives (mother/father and son/daughter), and indirect transmissions were those between second degree relatives (uncle/aunt and nephew/niece). The analyses were also performed on those members of G1 below and above the median that is the lower and upper 50% of G1. This was to identify the statistical phenomenon of regression to the mean, where anticipation may be demonstrated in the offspring of older parents (225). The following relationships were investigated:

1. The difference between each G1 and G2 pair was analysed. Tests for normality were performed (skewness and kurtosis) and confidence intervals were constructed around the median difference.

2. The median ages of G1 were compared to the median ages of G2. Medians rather than means were used and the Wilcoxon ranked sum test was preferred because the distributions of age of onset and death were non-Gaussian (206,207). The null hypothesis

was that the median age of G2 would be the same as that of G1. If anticipation is present, the median age of G2 will be lower than that of G1.

3. The number of occasions when G1 was greater than G2, i.e. positive anticipation, was analysed using a χ^2 test.

4. The numbers of instances of maternal and paternal inheritance were compared, in SOD1 positive and negative families. Contingency tables were constructed, comparing the values in our sample with expected values where there was no sex effect on inheritance (i.e. the number of paternal and maternal transmissions were equal), using a χ^2 test to determine whether or not there was a significant difference.

10.4 Results

A total of 160 first degree intergenerational transmissions were identified (Table 10.1). The mean age of death for all the individuals with ALS (n=503) was 55.2 years (95% confidence interval (CI) 54.0-56.4 years) with a mean disease duration of 3.5 years (CI 2.9-4.1). There was no significant difference ($p>0.05$) in age at death between patients with SOD1 mutations (53.9 years; CI 51.4 – 56.4; n=114) and those with no SOD1 mutation (55.6 years; CI 54.3 – 56.9; n=389). There was no significant difference ($p>0.05$) in duration of disease between patients with SOD1 mutations (4.9 years; CI 3.4 – 4.6; n=34) and those with no SOD1 mutation (3.3 years; CI 2.7 3.9, n= 193).

Table 10.1 Number of transmission for each relationship between G1 and G2 in families with ALS

Families	Father	Mother	Aunt	Uncle
All	123	149	124	108
SOD1	31	41	38	52
Non-SOD1	92	108	86	56

Age at death gave more complete data than age at onset or duration of disease, and was used for primary analysis. Three additional analyses were performed: comparisons were made between the different relationships of G1 to G2; comparisons were made between the upper, middle (i.e. between the 25 and 75 centiles) and lower 50% of G1 and their G2 relatives; and the SOD1 families were compared to the non-SOD1 families.

The median age of death was compared between the two generations (Table 10.2). For all transmissions, the Wilcoxon ranked sum test demonstrated significant increase in median age at death in G1 (55 years) than G2 (48 years) ($p<0.001$). This was significant irrespective of the SOD1 status or relationship of G1 to G2. The significance was present when studying the upper ($p<0.001$) and middle ($p<0.001$) 50% of the G1 range. When the lower 50% of G1 were studied there was no significant difference ($p>0.05$). Within the lower 50% of G1, there were 2 groups - indirect transmissions for all families and all transmissions for non-SOD1 families – where the differences were reversed, such that G2 was significantly older than G1.

When comparing the difference in age at death between first degree G1 and G2 pairs, the 95% confidence interval did not contain zero difference, indicating age of death in G1 was significantly older than G2 (Table 10.3), with the exception of all nonSOD1 individuals. Larger deviation from zero occurred when including only the older 50% of G1, and smaller deviations with the younger 50% members of G1.

Table 10.2 Median values of age at death for G1 and G2 in families with ALS with significance of difference between generations as determined by Wilcoxon ranked sum test.

	n	All samples		Upper 50% of G1		Middle 50% of G1		Lower 50% of G1	
		G1	G2	G1	G2	G1	G2	G1	G2
All families MFAU	291	55	48	63	54	55	48	45	45
		p<0.001		**p<0.001**		**p<0.001**		p>0.05*	
All families MF	160	56.5	52.5	64	54.5	56.5	53	49	48
		p<0.001		**p<0.001**		**p<0.005**		p>0.05*	
All families AU	131	53	46	62	48	53	45.5	42	43
		p<0.005		**p<0.001**		**p<0.001**		p<0.05*	
All families M	90	55	48.5	61	53	55	52	48	43
		p<0.005		**p<0.001**		**p<0.05**		p>0.05	
All families F	70	58	56	66	58	58	56	50	53
		p<.05		**p<0.001**		**p<0.05**		p>0.05*	
Non-SOD1 MFAU	184	55	48	62.5	58	55	49	42.5	47
		p<0.01		**p<0.001**		**p<0.05**		p<0.005*	
Non-SOD1 MF	110	56.5	51.5	63	53	56	52	50	50
		p<0.05		**p<0.001**		**p<0.01**		p>0.05*	
SOD1 MFAU	107	56	48	67	54.5	56	48	48	45
		p<0.001		**p<0.001**		**p<0.001**		p>0.05	
SOD1 MF	50	57	53.5	66	59.5	56	53.5	48	45
		p<0.05		**p<0.05**		p>0.05		p>0.05	

p values for Wilcoxon ranked sum test are shown in bold where G1 significantly greater than G2 (P<0.05)

* *G2>G1*

M=mother; F=father; U=uncle; A=aunt.

Table 10.3 Median difference in age at death between G1 and G2

	n	All samples		Upper 50% of G1		Middle 50% of G1		Lower 50% of G1	
		Median	CI	Median	CI	Median	CI	Median	CI
All families MFAU	291	+5	1.8	+12	2.3	+6	2.1	-2	2.3
All families MF	160	+6	2.3	+11	2.7	+6	2.9	-1	3.2
All families AU	131	+4.5	2.9	+13	3.6	+5	3.1	-2.5	3.2
All families M	90	+6	2.9	+8.5	4	+6	4.5	0	4.3
All families F	70	+4	3.8	+12	3.7	+2	3.9	-2	5.3
Non-SOD1 MFAU	184	+2	2.3	+13	2.7	+3.5	2.8	-4	2.8
Non-SOD1 MF	110	+6	2.9	+13	3.1	+5	3.4	-2	3.9
SOD1 MFAU	107	+8.5	2.9	+12	4	+7	3.8	+2	3.7
SOD1 MF	50	+7	3.9	+8	5	+4	5	+3	5.8

CI = 95% confidence interval. All samples tested for normality conform to a normal distribution.
M=mother; F=father; U=uncle; A=aunt.

When both first degree (mother and father) and second degree (aunt and uncle) modes of transmission were studied together, there was evidence of significant anticipation in age at death for all ALS subgroups when SOD1 families were analysed, but in the non-SOD1 families the confidence interval for all transmissions overlapped zero.

Frequency of positive (anticipation) and negative intergenerational differences were compared using a χ^2 test (Table 10.4). This demonstrates the phenomenon of regression to the mean, i.e. the older the members of G1, the more significant the tendency towards anticipation (Table 10.4). The expected result in a disease with no genetic anticipation would be equal numbers positive and negative results. χ^2 was positive for anticipation in all groups, with the exception of first degree intergenerational transfer in families with SOD1 mutations. The middle 50% of G1 in mothers, fathers and first degree SOD1 transmissions did not demonstrate a significant difference ($p>0.05$). When studying the younger 50% of G1, there was no significant difference, with the exception of all transmission in non-SOD1 families, where the tendency was against anticipation.

The number of instances of maternal and paternal inheritance was compared, in SOD1 positive and negative families. A χ^2 test revealed no difference in SOD1 positive ($p>0.1$) and SOD1 negative ($p>0.1$) cases for first degree transmissions. This suggests that parental sex has no effect on inheritance.

Table 10.4 Number of occasions when the difference between G1 and G2 is positive (anticipation) and negative. χ^2 and p values are given, for the null hypothesis that G1 = G2.

	n	All samples		Top 50% of G1		Middle 50% of G1		Bottom 50% of G1	
		+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
All families MFAU	291	182	109	117.5	22.5	99.5	48.5	64.5	86.5
		18.29		∞		17.56		3.21	
		p<0.001		**p<0.001**		**p<0.001**		p>0.05	
All families MF	160	101	59	66.5	13.5	52.5	31.5	34.5	45.5
		11.02		∞		5.25		1.51	
		p<0.001		**p<0.001**		**p<0.05**		p>0.05	
All families AU	131	81	50	55.5	9.5	41	23	25.5	40.5
		7.34		16.27		5.1		3.41	
		p<0.01		**p<0.001**		**p<0.05**		p>0.05	
All families M	90	56	34	34	9	29	16	20	23
		5.38		14.53		3.8		0.19	
		p<0.05		**p<0.001**		p>.05		p>0.05	
All families F	70	45	25	30.5	3.5	21	14	14.5	21.5
		5.71		21.36		1.4		1.36	
		p<0.05		**p<0.001**		p>.05		p>0.05	
Non-SOD1 MFAU	184	107.5	76.5	70	12	57.5	34.5	37.5	64.5
		5.22		∞		5.75		7.15	
		p<0.05		**p<0.001**		**p<0.05**		**p<0.01**	
Non-SOD1 MF	110	69.5	40.5	49	6	37.5	20.5	20.5	34.5
		7.65		∞		4.92		3.56	
		p<0.01		**p<0.001**		**p<0.05**		p>0.05	
SOD1 MFAU	107	74.5	32.5	45.5	8.5	45.5	18.5	29	25
		16.48		∞		11.39		0.296	
		p<0.001		**p<0.001**		**p<0.001**		p>0.05	
SOD1 MF	50	31.5	18.5	17.5	8.5	21.5	10.5	14	11
		3.38		4.00		3.78		0.36	
		p>0.05		**p<0.05**		p>0.05		p>0.05	

Significant p values are shown in bold. M=mother; F=father; U=uncle; A=aunt.

10.5 Discussion

The median ages of death and disease duration were similar to other studies of ALS (3). The median age at death in patients with familial ALS was significantly lower in the second generation of patients, $p<0.001$ (figure 10.1, table 10.2). The mean difference in age at death between the 2 generations was positive (younger death in second generation) and the number of occasions that positive anticipation occurred was significantly more than would be expected if there was no anticipation, $p<0.001$ (table 10.4).

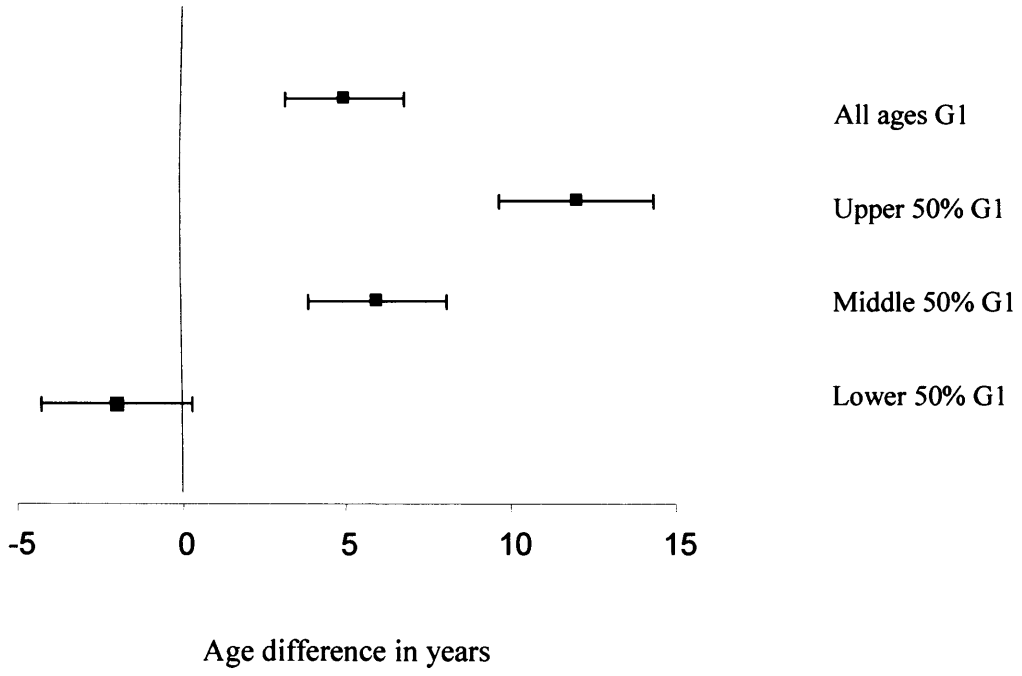


Figure 10.1 Median difference in age of death between G1 and G2 with 95% confidence interval for the upper, middle and lower 50% of age of G1.

Significant differences were observed in median age at death, mean differences in age at death, and frequency of earlier age at death, between the generations. These support the concept of anticipation in patients with ALS. Similar findings were present in

SOD1 and nonSOD1 groups (figure 10.2). It remains possible that a modifying gene may be active in both groups of patients, explaining variable penetrance in SOD1 and nonSOD1 families, and also possibly the occurrence of sporadic patients with SOD1 mutations (46). There are occasional instances of nonsignificance, which may be explained by incomplete ascertainment or insufficient numbers.

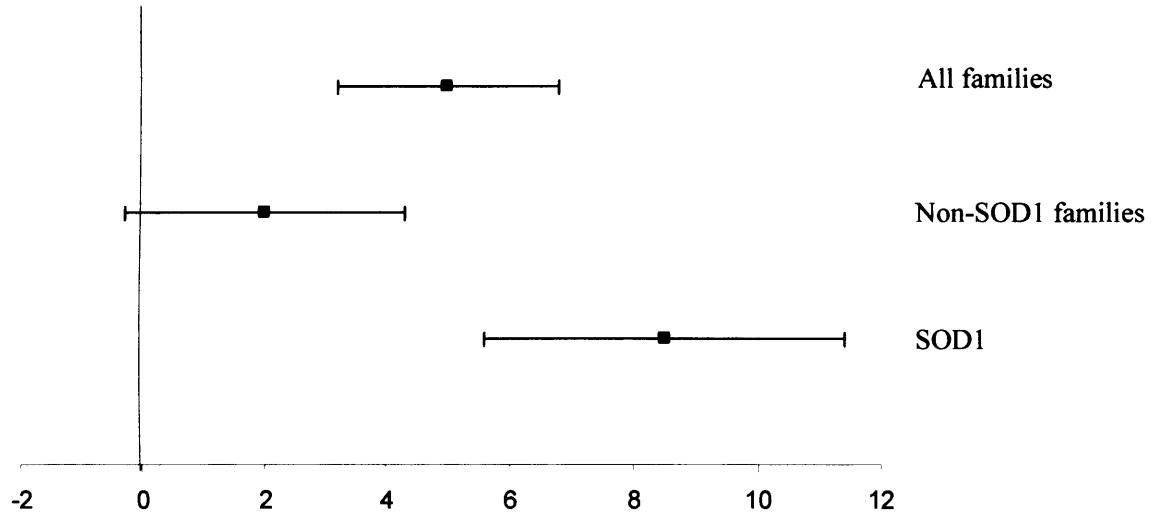


Figure 10.2 Median difference in age of death between G1 and G2 with 95% confidence interval for SOD1 and non-SOD1 families.

Ascertainment bias is recognised in studies of anticipation (226,227). Different key events have been used as the measured time point in studies of anticipation. Examples include age at onset in panic disorder (208) and schizophrenia (206), age at diagnosis in Crohn's disease (228) and age at onset of end stage renal failure in polycystic kidney disease (220). We used age at death as ALS is a rapidly progressive and fatal disease, and death is a definitive end-point. Death, for patients with ALS, occurs around 3 years after the onset of symptoms and is caused by ALS in over 98% of cases (229).

There is often incomplete information for age at onset, as it may be difficult for relatives and physicians to precisely recall or define the first feature of the disease. In some diseases, such as Crohn's disease, age at death may be an unreliable marker of disease, as this may not be the primary determinant of death. Improvements in diagnostic capability and the increased family awareness of the illness once a parent is diagnosed, could lead the offspring to be diagnosed much earlier than the parent (210,211). These forms of bias, which tend to suggest genetic anticipation when it is not actually present, are avoided by using age of death in ALS. The major factor, which could delay death in patients with ALS, is long term respiratory support. In this study, only one patient was identified to have received aggressive ventilatory support.

For various reasons, patients with a genetic disease may not wish or may be unable to have children. This process may lead to bias being introduced because of the effect on fertility or fitness. An individual with disease onset at a young age may be less likely to have children, either due to a wish to prevent others suffering with a similar disease, or because they would be physically incapable of looking after children. In a genetic line ending with mostly young onset patients, this will bias towards anticipation by eliminating many putative transmissions from early onset parents to late onset children. Other studies have used the second degree relatives as a means of minimising this bias. For example, this has been used in schizophrenia (206). Our analysis of second degree relatives supported the presence of anticipation.

Ascertainment bias may occur if not all individuals in the study are interviewed at the same age. Anticipation may be introduced if offspring are interviewed at a younger age. This could be due to simultaneous onset in both generations attracting the differing attention of family members and their physicians. This would present as apparent later onset in parents and earlier onset in offspring. G2 would have an earlier onset than G1 and favour anticipation. This is further compounded, as any siblings of G2, who may eventually develop the disease, thus negating the effect of the perceived anticipation, would not be detected at the time of data collection.

Huntington's disease was found to have only weak evidence of anticipation in the female line (230). This finding was consistent with regression to the mean such that

anticipation was only seen in the children of older mothers (231). Despite this weak association Huntington's disease was shown to be due to a trinucleotide repeat expansion, which could be transmitted by the female line (232). It has been reported that only around 70% of the variability of age at onset in Huntington's disease is attributed to the CAG repeat size. A significant additional contribution to the variability is attributed to a TAA repeat polymorphism, related to the kainate receptor GluR6 on chromosome 6 (227,233).

With no anticipation, the median value of G1 minus G2 will be close to zero, with confidence intervals crossing the zero axis. Regression to the mean is a phenomenon whereby higher values of G1 artificially increase the degree of perceived anticipation, and *vice versa* for lower values of G1. Such a tendency causes the value to be positive when selecting the oldest 50% of G1 and negative when selecting the youngest 50% of G1. This phenomenon was observed when the data were analysed in this way. This leads me to interpret the suggestion of anticipation in this analysis with caution. This does not however exclude anticipation or unstable DNA disease (207,225,231).

This study does not demonstrate maternal inheritance in familial ALS, even without attempted correction for potential disputes of paternity or families where paternal contact has been lost. The lack of a sex effect in inheritance occurs in SOD1 positive and negative families, implying that mitochondrial transmission, alone, does not account for inheritance.

In conclusion, although there is apparent anticipation in age at death between generations in patients with familial ALS, there remains the difficulty of fully accounting for ascertainment bias, as illustrated by the failure to demonstrate anticipation in the offspring of the younger 50% of the patient population studied. The results may be important in pointing to underlying genetic mechanisms to explain the multifactorial predispositions, genetic and environmental, to developing ALS. There is no evidence of maternal inheritance in the sample studied. This would suggest that abnormalities in mtDNA are not inherited in familial ALS.

11 Conclusions

11.1 Summary of Results

The findings of this study are, in summary:

1) There are no specific histological changes in the skeletal muscle of patients with ALS (chapter 4).

2) Mitochondrial respiratory chain activity is the same in the skeletal muscle homogenate of controls and patients with ALS (chapter 5).

3) Aconitase activity is the same in the skeletal muscle of controls and patients with ALS (chapter 5), suggesting that the levels of oxidative damage are the same (chapter 5).

4) Mitochondrial respiratory chain function is the same in cultured myoblasts, fibroblasts and platelet-derived cybrids from controls and patients with ALS (chapter 6).

5) The expression of the mitochondrially encoded protein, subunit I of complex IV is the same in fibroblasts from controls and patients with ALS (chapter 7).

6) Total levels of mtDNA, mtDNA deletions and rearrangements are the same in the skeletal muscle of controls and patients with ALS (chapter 8).

7) Aconitase activity is more affected by exposure to paraquat in fibroblast and myoblast cultures from patients with ALS than cell cultures from controls, suggesting that patient cultures are more susceptible to oxidative stress. This is not seen in cultures of platelet derived cybrids (chapter 9).

8) Caspase 3 activity is more stimulated by exposure to paraquat in fibroblast and myoblast cultures from patients with ALS than cell cultures from controls,

suggesting that patient cultures are more susceptible to oxidative stress. This is not seen in cultures of platelet derived cybrids (chapter 9).

9) There is no convincing evidence for anticipation or sex-effect in the inheritance of familial ALS (chapter 10).

11.2 Discussion

The body of evidence implicating mitochondrial abnormalities in the remote tissues of patients with ALS is broad, inconsistent and sometimes contradictory. Most groups have tended to focus on one particular tissue or cell type in their analysis. Whole muscle analysis has been reported as showing mitochondrial abnormalities in terms of respiratory chain complex defects (98) and metabolic defects (91). Other groups report no differences in mitochondrial activities between controls and patients (104) when studying respiratory function and ATP synthesis. Analysis of platelet-derived cybrids was reported as demonstrating a difference in respiratory chain complex activity and calcium homeostasis by one group (120) but another group demonstrated that the mtDNA from ALS patients' platelets restores normal respiratory function in ρ^0 cells (122). Fibroblast and myoblast cell culture analysis has generally not been carried out with the exception of a small study looking at the susceptibility of fibroblasts from ALS patients to oxidative stress (56). This study demonstrated that SOD1-FALS and SALS fibroblasts were significantly more sensitive than controls to the free radical generating agent, SIN-1 but not to serum withdrawal. SOD1-FALS fibroblasts were more sensitive to H_2O_2 than SALS fibroblasts and than control fibroblasts.

The analysis of mtDNA shows similar disparity. Whereas some groups have identified increased levels of deletions and lower total amounts of mtDNA in tissues taken from patients (112,115) others have shown no differences in mtDNA between patients and controls (116).

Even without consideration of the potential publication bias against "normative" data (234) in the study of mitochondria and ALS, there does not appear to be a clear

consensus on the status of mitochondrial abnormalities in remote tissues. It was for this reason that this study sought to analyse a number of different tissues using different experimental paradigms across the same tissue and cell types.

Initial work, looking at the appearances of skeletal muscle from ALS patients on light microscopy (chapter 4) demonstrated that fibre changes consistent with a denervation – reinervation pattern were the only changes positively associated with a diagnosis of ALS. There were no changes suggestive of classical mitochondrial disease (ragged-red fibres or COX negative fibres) and there was not a significant degree of inflammatory change.

Analysis of mitochondrial respiratory chain function was normal in muscle, platelet derived cybrids, fibroblasts and myoblasts from patients with ALS (Chapters 5 and 6). There were no consistent significant differences between patients, controls or disease controls in any of the tissues studied. While this finding is at odds with some of the previously published work (103,120), it does confirm the conclusion of others (116,122). The possible reasons that these differences may have emerged is discussed below.

The single cell analysis of myoblast and fibroblast cultures from patients and controls demonstrated no mtDNA protein expression abnormalities in the cultures (Chapter 7). This is consistent with the finding of normal respiratory complex activity in the cells, and is also against the concept that random systemic mtDNA mutations occur with a higher frequency in patients with ALS. The relative lack of documented cases of ALS resulting from discrete mtDNA mutations would also support this (99). Invoking mtDNA abnormalities to explain the primary pathogenesis of ALS is not borne out by the analysis of the inheritance of non-SOD1 familial ALS (Chapter 10). If mitochondrial inheritance was responsible for even a portion of non-SOD1 cases, it would be expected that there would be a sex bias in inheritance, but this was not the case. Cellular mitochondrial abnormalities have been described in association with some of the trinucleotide repeat sequence disorders (106) which demonstrate anticipation. Analysis of familial ALS cases show that any perceived anticipation is likely to represent the statistical phenomenon of regression to the mean.

The normal mitochondrial protein expression and normal respiratory chain function in the remote tissues of patients with ALS are found in association with normal total amounts and normal levels of deleted mtDNA in these tissues (chapter 8). The experimental findings are, therefore, consistently against a systemic primary mtDNA abnormality in ALS. The single patient that was included in the study with an SOD1 mutation did not demonstrate different mitochondrial DNA expression or metabolic activity to the sporadic cell cultures and tissues which were analysed. Although this was a single patient, it does indicate that the metabolic profiles of systemic tissues may be the same for sporadic and familial patients, hence the similarities seen in the phenotypes of each disease.

There is the possibility that apparent mitochondrial abnormalities seen in SOD1 transgenic mice, cell models and human tissues may represent secondary rather than primary manifestations of an underlying pathogenic process. This supposition is supported by a recent study which shows that motor neuron cells expressing low levels of mutant SOD1 are in a chronic state of oxidative stress and mitochondrial dysfunction (as measured by [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) conversion rates (235). Given that studies on human tissues are generally performed on patients with established ALS, it is difficult to draw the conclusion that mitochondrial abnormalities are the primary pathogenic process in these individuals.

The finding that remote tissues from patients with ALS are more susceptible to experimental oxidative stress, in terms of apoptotic activity and aconitase inhibition, concurs with published work on the sensitivity of patient fibroblasts to oxidative stress (56), as well as studies which show a greater amount of apoptotic activity in response to oxidative stress in transgenic SOD1 mice (198). Neuronal cell models transfected with mutant SOD1 show a greater death rate in response to oxidative stress than cells transfected with normal SOD1 (79) as impairment of glutamate transport mechanisms (82). It would appear that the mutant SOD1 found in familial forms of ALS may exert its toxic effect by lowering the cell's resistance to oxidative stress. By extension, the underlying pathological mechanism of neuronal death in sporadic ALS would involve a similar mechanism in view of the relative phenotypic homogeneity between the two

diseases. The present study confirms that there is an increased sensitivity to oxidative stress in two different remote cell cultures from patients with the sporadic disease. Thorough analysis of mitochondrial function suggests that the cause of this increased sensitivity is not of mitochondrial origin, especially in view of the normal levels of oxidative stress and apoptosis seen in patient platelet-derived cybrid cell cultures.

It is of note that the tissue respiratory chain enzyme activities, mtDNA analysis and susceptibibilty to oxidative stress revealed no consistent differences between the tissue and cell samples taken from the patient with familial disease and those with sporadic ALS. Although this is a single case, it does indicate that there are unlikely to major differences in the cellular pathology of the sporadic and familial forms of ALS, further work with a greater number of defined familial samples would obviously be required to confirm this supposition.

Given that mtDNA is more sensitive to oxidative damage than nuclear DNA (26), the findings of abnormal mitochondrial activity in muscle (103) and cybrid cultures (120) of patients with ALS that other groups have identified may represent the secondary effects of oxidative damage rather than a primary pathological process. The conditions of cell culture and tissue analysis vary between centres, and if cell lines or tissue were grown or stored in conditions where higher levels of oxidative stress were encountered, detrimental effects on mitochondrial activity may occur. This would explain why other groups identify no abnormalities in remote tissues of patients with ALS (104,116,122). With the consistency of normal results through the range of different paradigms and different tissues that the present study has analysed for mitochondria, this appears a plausible theory. The supplementation of growth medium with foetal calf serum introduces another inter-centre variable, because the composition of the sera varies between manufacturers and batches. A study looking at mitochondrial enzyme activity, histochemical abnormalities and mtDNA levels identified no significant differences in muscle biopsies taken from patients with ALS, disease controls and normal controls (117). The findings from this comprehensive analysis are consistent with those from this study.

Given the relative size and metabolic activity of motor neurons, such cells would be expected to sustain a greater degree of oxidative stress throughout life. With the poor regenerative potential of such tissues in adulthood, this stress would translate into oxidative damage more readily than for other tissues. The threshold effect for damage and the development of overt pathology would be lower in patients with ALS. The present study suggests that this is due to a systemic factor which increases susceptibility to such damage. These findings are potentially important for a number of reasons:

- The investigation of the pathogenesis of sporadic ALS may be possible by using remote cell cultures from living patients.
- A model of sporadic ALS may be developed using these cultures. This avoids the need to draw conclusions about the human disease from mouse or cell SOD1 transgenic models.
- The potential assessment of therapeutic agents in limiting oxidative stress in such cultures may be possible.
- The particular properties of remote cell cultures may be utilized to provide earlier diagnostic tools.

11.3 Further Work

The replication of these findings in cell cultures taken from transgenic mice, or from neuronal cultures would be a potentially valuable method of determining the common pathological pathways in familial and sporadic ALS. A larger study with a greater number of familial patients would also be an appropriate means of further investigating these ideas.

In order to confirm the possibility that mitochondrial abnormalities in the cell cultures of patients with ALS may be a secondary effect, there would be some value in carrying out respiratory chain assays and protein expression estimation in different cell cultures that had been subject to different levels of such stress.

The analysis of primary tissue and cell cultures from large numbers of patients at different points of progression of the disease would also go some way to addressing the question of cellular abnormalities manifesting as a secondary effect of the disease process.

Carrying out experiments using these paradigms in patients with known mitochondrial diseases would allow comparisons to be made with neurodegenerative conditions where these experimental techniques have previously been utilised.

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Appendix 1 – Media and buffer composition

Cybrid medium

500ml Dulbecco's Modified Eagle Medium

50ml dialysed foetal calf serum (final conc: 10%)

2.5 ml 50 U/ml penicillin 2.5 ml 50 U/ml streptomycin (aliquoted together)

Denhardt's Solution

2% (w/v) BSA

2% (w/v) Ficol 400

2% (w/v) polyvinyl pyrrolidone (PVP).

Fibroblast / ρ^0 medium

500 ml Dulbecco's Modified Eagle Medium (Gibco)

50 ml foetal calf serum (Biosciences) (final conc: 10%)

1 ml 2 mM uridine

5 ml 1 mM sodium pyruvate

2.5 ml 50 U/ml penicillin 2.5 ml 50 U/ml streptomycin (aliquoted together)

Homogenisation Buffer

0.25 M Sucrose

10 mM Tris.HCl (pH 7.4)

1 mM Potassium EDTA

Myoblast dissociation solution

10 ml Dulbecco's Modified Eagle Medium

12000 U Collagenase (Sigma)

3.2 ml of 0.1% trypsin in versene solution

40 mg bovine serum albumin (Sigma)

- Dissolved and filtered through a 2 μm filter (Sarstedt).

Myoblast medium

500 ml Dulbecco's Modified Eagle Medium

100 ml foetal calf serum (final conc: 20%)

1 ml 2 mM uridine

5 ml 1 mM sodium pyruvate

2.5 ml 50 U/ml penicillin 2.5 ml 50 U/ml streptomycin (aliquoted together)

PBS

10 mM sodium phosphate

0.9% NaCl

pH 7.4

SSC Buffer

0.15 M NaCl

0.015 M sodium citrate

pH 7.0

TAE Buffer

20 mM Tris-acetate

1 mM Sodium EDTA

0.2 M Acetic Acid

pH 7.4

TE Buffer

10 mM Tris.HCl

1 mM Sodium EDTA

pH 7.4

Trypsin Solution (0.1%)

4ml 2.5% of trypsin stock solution (Gibco) in 100ml versene solution (1:5000; Gibco)

Tyrode's Buffer

150 mM NaCl

5 mM HEPES

0.55 mM NaH_2PO_4

7 mM $NaHCO_3$

2.7 mM KCl

5.6 mM Glucose

1 mM Potassium EDTA

pH 7.4

1 mM $MgCl_2$

Appendix 2 – Data tables

Appendix 2(a) – Normal (control) muscle mitochondrial enzyme values for all samples assayed. Activities per citrate synthase activity, protein concentrations and activity as a ratio of protein concentration are shown. There was insufficient material in samples (C6, C15, C17) to perform protein assays.

	Activity / CS activity			**Protein**	**Activity / Protein Concentration**		
	I	II/III	IV	(mg/ml)	I *	II/III *	IV **
C2	0.361	0.064	0.076	2.440	27.800	44.822	10.805
C3	0.107	0.210	0.038	2.340	25.157	47.412	14.982
C5	0.158	0.259	0.023	1.520	36.670	19.003	22.566
C6	0.129	0.208	0.041	X	X	X	X
C7	0.100	0.073	0.043	3.880	59.485	15.025	6.260
C8	0.099	0.163	0.053	3.980	24.337	39.894	12.707
C9	0.113	0.259	0.018	1.490	22.873	31.371	10.645
C11	0.262	0.133	0.052	1.630	23.538	42.477	11.201
C12	0.120	0.120	0.033	3.880	35.920	26.222	15.446
C14	0.139	0.080	0.032	3.370	14.827	24.411	13.400
C15	0.053	0.250	0.011	X	X	X	X
C16	0.358	0.084	0.035	3.960	17.964	21.515	7.086
C17	0.087	0.157	0.041	X	X	X	X
C18	0.367	0.235	0.039	3.580	29.082	39.987	12.178
C19	0.164	0.204	0.032	1.850	48.900	31.312	5.196
C20	0.160	0.220	0.067	1.160	39.783	22.897	10.624
C21	0.119	0.143	0.047	1.700	58.046	58.451	11.813

* $nmoles.min^{-1}.mg^{-1}$ ** $k.min^{-1}.mg^{-1}$. X = insufficient sample to process.

Appendix 2(b) – Disease control muscle mitochondrial enzyme values for all samples assayed. Activities per citrate synthase activity, protein concentrations and activity as a ratio of protein concentration are shown.

	Activity / CS activity			**Protein**	**Activity / Protein Concentration**		
	I	II/III	IV	(mg/ml)	I*	II/III*	IV**
D1	0.263	0.190	0.063	2.410	44.087	44.522	7.102
D3	0.177	0.223	0.022	2.120	28.529	35.943	13.485
D4	0.109	0.308	0.034	1.900	47.323	40.260	11.963
D5	0.115	0.140	0.017	1.500	56.289	34.470	12.854
D6	0.202	0.132	0.016	2.600	47.815	24.274	17.691
D7	0.260	0.350	0.032	2.000	13.064	14.663	5.821
D8	0.234	0.226	0.033	3.700	26.060	39.680	16.463
D9	0.134	0.186	0.051	3.100	11.648	12.560	12.195
D10	0.268	0.180	0.026	5.200	11.223	13.620	5.253
D11	0.243	0.170	0.039	4.090	40.697	54.785	5.009
D12	0.203	0.205	0.033	1.900	42.736	52.281	12.737

* $nmoles.min^{-1}.mg^{-1}$ ** $k.min^{-1}.mg^{-1}$.

Appendix 2(c) – Patient (disease) mitochondrial enzyme muscle values for all samples assayed. Activities per citrate synthase activity, protein concentrations and activity as a ratio of protein concentration are shown.

	Activity / CS activity			**Protein**	**Activity / Protein Concentration**		
	I	II/III	IV	(mg/ml)	I*	II/III*	IV**
PA1	0.198	0.137	0.035	5.350	19.380	22.647	3.831
PA2	0.131	0.093	0.039	2.130	31.689	41.330	10.559
PA3	0.172	0.201	0.034	2.030	26.419	18.756	7.865
PA4	0.152	0.156	0.048	3.160	43.037	58.950	16.037
PA5	0.241	0.163	0.057	4.430	16.696	23.678	10.777
PA6	0.140	0.230	0.035	1.980	44.935	24.762	22.813
PA7	0.110	0.156	0.071	3.450	37.139	12.998	7.525
PA8	0.520	0.182	0.051	2.870	36.861	52.200	15.833
PA9	0.270	0.380	0.022	2.160	42.978	29.068	10.094
PA10	0.260	0.144	0.044	3.660	57.408	58.790	5.021
PA11	0.340	0.201	0.050	3.040	37.076	21.951	5.452
PA12	0.270	0.237	0.050	1.840	44.814	40.663	X

* nmoles.min^{-1}.mg^{-1} **k.min^{-1}.mg^{-1}. X = insufficient sample to process.

Appendix 2(d) – Muscle absolute aconitase activities for all normal (control) samples shown with corrected values for sample protein concentrations.

Sample	Uncorrected Aconitase Activity (mmol/min/ml)	Protein Concentration (mg/ml)	Corrected Activity (mmol/min/mg)
C2	14.48	1.64	8.83
C3	29.70	3.88	7.66
C7	10.20	3.37	3.03
C8	60.72	3.96	15.33
C9	78.59	3.58	21.95
C11	2.37	1.84	1.29
C12	33.72	2.44	13.82
C14	12.91	1.15	11.23
C15	46.20	1.16	39.73
C18	15.18	2.34	6.49
C19	64.36	3.53	18.23
C20	37.94	3.98	9.53
C21	31.11	2.49	12.49

Appendix 2(e) – Muscle absolute aconitase activities for all disease control samples shown with corrected values for sample protein concentrations.

Sample	Uncorrected Aconitase Activity (mmol/min/ml)	Protein Concentration (mg/ml)	Corrected Activity (mmol/min/mg)
D1	6.61	2.40	2.75
D3	34.86	0.90	38.73
D4	21.60	2.12	10.19
D5	3.32	0.73	4.55
D6	36.49	1.50	24.32
D7	25.42	1.13	22.50
D12	48.05	5.00	9.61

Appendix 2(f) – Muscle absolute aconitase activities for all patient (ALS) samples shown with corrected values for sample protein concentrations.

Sample	Uncorrected Aconitase Activity (mmol/min/ml)	Protein Concentration (mg/ml)	Corrected Activity (mmol/min/mg)
PA1	24.24	5.35	4.53
PA2	63.75	3.40	18.75
PA4	35.46	3.04	11.66
PA5	21.09	0.62	34.02
PA7	13.52	4.50	3.00
PA8	27.63	1.12	24.67
PA9	24.54	4.43	5.54
PA10	9.89	3.48	2.84
PA11	17.10	1.10	15.55
PA12	31.72	2.16	14.67

Appendix 2(g) – Myoblast mitochondrial respiratory chain specific activities for control (C), disease control (D) and patient (PA) samples.

	Complex activities as a Ratio of Citrate Synthase Activity		
Sample	**I**	**II/III**	**IV**
C2	0.129	0.164	0.052
C4	0.160	0.259	0.072
C5	0.285	0.267	0.058
C6	0.086	0.297	0.025
C7	0.270	0.440	0.050
C12	0.185	0.228	0.056
C14	0.215	0.244	0.014
C15	0.138	0.212	0.042
C17	0.146	0.287	0.078
C19	0.104	0.307	0.046
C20	0.100	0.491	0.031
D3	0.142	0.228	0.040
D4	0.198	0.270	0.006
D5	0.100	0.278	0.030
D6	0.176	0.296	0.087
D7	0.080	0.328	0.029
D8	0.301	0.359	0.022
PA1	0.119	0.251	0.043
PA2	0.243	0.276	0.018
PA3	0.092	0.087	0.008
PA4	0.062	0.498	0.053
PA5	0.152	0.212	0.045
PA6	0.150	0.158	0.019
PA8	0.083	0.255	0.030
PA10	0.142	0.310	0.037
PA11	0.269	0.193	0.025
PA12	0.220	0.360	0.050

Appendix 2(h) – Fibroblast mitochondrial respiratory chain specific activities for control (C), disease control (D) and patient (PA) samples.

	Complex Activities as a Ratio of Citrate Synthase Activity		
Sample	**I**	**II/III**	**IV**
C2	0.149	0.157	0.042
C3	0.063	0.181	0.043
C4	0.174	0.120	0.026
C5	0.100	0.255	0.033
C6	0.263	0.157	0.028
C11	0.090	0.328	0.082
C12	0.262	0.202	0.056
C18	0.250	0.330	0.046
C19	0.093	0.324	0.014
D3	0.163	0.258	0.053
D5	0.386	0.200	0.009
D6	0.228	0.233	0.014
D7	0.170	0.316	0.058
D8	0.186	0.248	0.053
PA3	0.169	0.209	0.039
PA4	0.176	0.152	0.043
PA5	0.105	0.199	0.052
PA6	0.073	0.307	0.088
PA7	0.079	0.209	0.048
PA8	0.077	0.232	0.032
PA9	0.252	0.383	0.070
PA10	0.144	0.280	0.040

Appendix 2(i) – Platelet-derived cybrid mitochondrial respiratory chain specific activities for control (C), disease control (D) and patient (PA) samples.

	Complex Activities as a Ratio of Citrate Synthase Activity		
Sample	**I**	**II/III**	**IV**
C21	0.498	0.328	0.016
C9	0.283	0.193	0.073
C18	0.397	0.304	0.094
C19	0.275	0.296	0.074
C20	0.270	0.270	0.074
C14	0.151	0.407	0.055
D1	0.580	0.240	0.033
D6	0.254	0.286	0.034
D8	0.244	0.350	0.038
PA7	0.195	0.415	0.069
PA2	0.315	0.400	0.068
PA3	0.206	0.127	0.028
PA4	0.199	0.460	0.041
PA10	x	0.313	0.088
PA11	0.320	0.440	0.050
PA12	0.650	0.350	0.070

X = insufficient sample to process.

Appendix 2(j) – mtDNA and nDNA signals of control (C), disease control (D) and patient (PA) skeletal muscle samples determined by phosphoimaging of ^{32}P-signals after Southern blot hybridiasation along with the number of bands seen on lrPCR for each sample.

Sample	mtDNA Signal	nDNA Signal	Ratio mtDNA/nNA	Number of Bands
C3	36354	26227	1.39	x
C4	x	x	x	6
C8	244884	255555	0.96	9
C9	x	x	x	6
C11	94612	62987	1.50	1
C12	57505	58408	0.98	1
C14	122132	230626	0.53	8
C14	180546	190867	0.95	8
C15	57983	112661	0.07	1
C16	x	x	x	1
C17	40425	43692	0.93	1
C18	54157	48044	1.13	1
C19	72320	54528	1.33	2
C20	58266	45533	1.28	x
C21	118319	154756	0.76	x
D1	x	x	x	1
D3	178898	103498	1.73	1
D4	x	x	x	1
D5	x	x	x	1
D6	119805	141568	0.85	5
D7	77262	74999	1.03	1
D11	x	x	x	2
D12	x	x	x	1
PA1	130592	114765	1.14	5
PA2	86744	167345	0.52	7
PA3	49381	33979	1.45	1
PA4	30427	53634	0.57	13
PA5	102651	81548	1.26	6
PA6	98523	111638	0.88	x
PA7	57430	44775	1.28	8
PA8	131555	Air bubble	x	1
PA9	49781	38022	1.31	1
PA10	30038	32123	0.94	x
PA11	91209	47786	1.91	1
PA12	107055	129315	0.83	x

X = insufficient sample to process.

Appendix 2(k) Absolute aconitase activity per mg of protein measured in control (C), disease control (D) and patient (PA) cybrids grown at different paraquat concentrations. The percentage fall in aconitase activities between different concentrations is shown.

	Aconitase Activity at Different Paraquat Concentrations (mmol.min^{-1}.mg^{-1})			**% Fall in Aconitase Activity**	
Sample	0 mM	0.1 mM	1 mM	0 versus 0.1 mM	0 versus 1 mM
C14	6.2	1.2	0.03	80.7	99.5
C18	4.6	1.0	0.3	79.1	94.1
C19	3.4	0.7	0	80.0	100
C20	12.7	1.6	0	87.4	100
C21	5.5	0.3	0	94.5	100
D1	9.4	0.4	0	95.4	100
D6	5.6	0.5	0	90.9	100
D8	10.9	0.9	0	92.1	100
PA2	7.9	0.2	0	97.6	100
PA3	13.0	4.1	0	68.5	100
PA4	5.9	0.5	0	91.5	100
PA7	3.7	0.6	0	84.1	100
PA10	3.0	0.1	0	97.0	100
PA11	6.2	1.5	0	75.8	100
PA12	13.5	4.5	0	66.7	100

Appendix 2(l) Absolute aconitase activity per mg of protein measured in control (C), disease control (D) and patient (PA) myoblasts grown at different paraquat concentrations. The percentage fall in aconitase activities between different concentrations is shown.

	Aconitase Activity at Different Paraquat Concentrations ($mmol.min^{-1}.mg^{-1}$)				**% Fall in Aconitase Activity**		
Sample	0mM	0.05 mM	0.1mM	1 mM	0 versus 0.05 mM	0 versus 0.1 mM	0 versus 1 mM
C5	1.9	0.6	0.0	0.0	70.5	100	100
C6	1.5	1.0	1.0	0.3	33.3	34.0	80.7
C7	2.2	1.9	1.0	0	14.8	55.2	100
C8	6.0	2.9	1.2	0	50.6	79.8	100
C14	1.5	0.5	0.9	0	65.3	43.3	98.7
C17	3.7	0.8	0.2	0	77.4	95.4	100
C18	6.4	4.8	2.6	0	25.0	59.4	90.6
C21	1.9	1.0	0.6	0	47.4	69.5	100
D3	3.3	0.9	0.4	0	72.5	89.3	100
D4	7.2	3.3	0.7	0	54.0	89.8	100
D6	7.2	0.7	0.6	0.4	90.7	91.8	95.1
D7	5.7	2.1	1.5	1.0	63.2	73.3	82.5
D8	6.8	2.2	1.4	0.8	67.1	79.4	88.2
PA1	8.4	1.7	0.9	0	80.1	89.3	100
PA2	2.3	0.4	0.0	0	81.3	100	100
PA3	3.0	1.7	0.1	0	43.9	97.0	100
PA4	4.6	0.9	0.0	0	80.4	100	100
PA6	4.2	2.3	1.1	0.1	45.2	73.8	97.4
PA8	5.1	2.3	0.1	0	54.9	98.0	100
PA9	6.0	2.3	1.6	1.1	61.9	73.5	82.1
PA10	2.6	0.8	0.2	0	70.2	91.4	100
PA11	2.3	0.4	0.3	0	83.6	85.8	100
PA12	5.0	1.6	0.1	0	68.0	98.8	100

Appendix 2(m) Absolute aconitase activity per mg of protein measured in control (C), disease control (D) and patient (PA) fibroblasts grown at different paraquat concentrations. The percentage fall in aconitase activities between different concentrations is shown.

	Aconitase Activity at Different Paraquat Concentrations ($mmol.min^{-1}.mg^{-1}$)			% Fall in Aconitase Activity	
Sample	0mM	0.1mM	1mM	0 versus 0.1 mM	0 versus 1 mM
C2	1.6	1.0	0.1	39.1	91.3
C3	1.2	0.2	0.0	81.1	97.1
C4	2.0	0.6	0.1	69.8	95.0
C5	2.1	1.1	0.1	47.9	94.2
C	2.0	0.2	0	89.3	100
C11	2.7	0.3	0.3	90.6	90.6
C18	2.6	0.8	0	69.2	100
D3	3.1	0.6	0.1	80.3	97.1
D5	0.7	0.3	0.1	56.9	92.2
D6	0.7	0.2	0	79.7	100
D7	1.2	0.1	0	90.0	100
D8	1.7	0.2	0	87.2	100
PA3	1.4	0.1	0	93.8	100
PA4	0.8	0.0	0	100.0	100
PA6	1.1	0.1	0	91.1	100
PA7	1.5	0.3	0.1	83.1	96.1
PA8	2.8	0.8	0	70.7	98.6
PA9	1.1	0.1	0	90.8	100

Appendix 2(n) Relative proportions of apoptotoic (caspase-activated) cells and unaffected cells in control (C), disease control (D) and patient (PA) cybrid cultures grown in different paraquat concentrations. The percentage change in the levels of apoptosis seen between different concentrations of paraquat is also shown.

	Ratio of Apoptotic to Non-apoptotic Cells Seen in Different Paraquat Concentrations			**% Change in Apoptosis Levels**	
Sample	0 mM	0.1 mM	1 mM	0 versus 0.1 mM	0 versus 1 mM
C14	0.07	0.15	0.36	112.86	411.43
C18	0.00	0	0.17	0	165.00
C19	0.04	0.10	0.06	140.00	50.00
C20	0.01	0.01	0.01	14.29	28.57
C21	0.06	0.08	0.07	40.35	26.32
D1	0.03	0.04	0.09	61.54	242.31
D6	0.06	0.07	0.29	4.69	348.44
D8	0.17	0.11	0.16	-36.47	-5.88
PA2	0.05	0.06	0.08	11.11	44.44
PA3	0.07	0.17	0.25	142.86	251.43
PA4	0.02	0.04	0.03	100.00	50.00
PA7	0.00	0.01	0	7.00	37.00
PA10	0.06	0.09	0.12	50.00	100.00
PA11	0.01	0.01	0.04	44.00	311.00
PA12	0.04	0.02	0.02	-43.24	-43.24

Appendix 2(o) Relative proportions of apoptotoic (caspase-activated) cells and unaffected cells in control (C), disease control (D) and patient (PA) myoblast cultures grown in different paraquat concentrations. The percentage change in the levels of apoptosis seen between different concentrations of paraquat is also shown.

	Ratio of Apoptotic to Non-apoptotic Cells Seen in Different Paraquat Concentrations			**% Change in Apoptosis Levels**	
Sample	0 mM	0.1 mM	1mM	0 versus 0.1 mM	0 versus 1 mM
C2	0.08	0.09	0.10	14.63	17.07
C5	0.11	0.14	0.29	27.27	163.64
C6	0.06	0.11	0.10	96.43	78.57
C7	0.05	0.13	0.10	150.94	88.68
C16	0.06	0.18	0.14	195.08	129.51
C17	0.12	0.14	0.19	16.67	58.33
C18	0.17	0.20	0.13	17.65	-23.53
C20	0.11	0.16	0.29	45.45	163.64
C21	0.14	0.17	0.20	21.43	42.86
D5	0.07	0.11	0.15	57.14	114.29
D6	0.05	0.16	0.17	220.00	240.00
D7	0.13	0.13	0.17	3.17	34.92
D8	0.21	0.36	0.30	71.43	42.86
PA1	0.07	0.17	0.25	142.86	257.14
PA2	0.10	0.33	0.49	230.00	390.00
PA3	0.09	0.10	0.20	6.38	112.77
PA4	0.06	0.12	0.18	118.18	227.27
PA6	0.16	0.30	0.43	87.50	168.75
PA7	0.13	0.30	0.53	130.77	307.69
PA8	0.14	0.23	0.27	64.29	92.86
PA10	0.07	0.32	0.39	338.36	434.25
PA11	0.07	0.23	0.24	228.57	242.86

Appendix 2(p) Relative proportions of apoptotoic (caspase-activated) cells and unaffected cells in control (C), disease control (D) and patient (PA) fibroblast cultures grown in different paraquat concentrations. The percentage change in the levels of apoptosis seen between different concentrations of paraquat is also shown.

	Ratio of Apoptotic to Non-apoptotic Cells Seen in Different Paraquat Concentrations			**% Change in Apoptosis Levels**	
Sample	0 mM	0.1 mM	1 mM	0 versus 0.1 mM	0 versus 1 mM
C2	0.25	0.30	0.40	20.00	60.00
C3	0.02	0.02	0.03	-21.26	42.86
C4	0.13	0.31	0.15	137.42	14.81
C5	0.04	0.02	0.06	-46.43	40.45
C6	0.05	0.07	0.06	27.71	4.04
C7	0.24	0.33	0.28	37.50	16.67
C11	0.12	0.13	0.15	9.17	25.00
C18	0.53	0.08	0.58	-84.91	9.43
C19	0.09	0.11	0.39	18.89	333.33
D3	0.06	0.02	0.08	-63.53	53.48
D5	0.09	0.14	0.15	55.56	66.67
D6	0.26	0.19	0.38	-26.92	46.15
D7	0.18	0.05	0.40	-71.43	128.57
D8	0.15	0.32	0.34	113.33	126.67
PA3	0.10	0.20	0.40	100.00	300.00
PA4	0.06	0.04	0.07	-28.57	24.62
PA7	0.06	0.04	0.12	-36.05	91.52
PA6	0.25	0.68	0.67	172.00	168.00
PA9	0.34	0.72	0.92	111.76	170.59
PA8	0.05	0.10	0.13	100.00	150.00
PA10	0.07	0.13	0.16	85.71	128.57

Appendix 3 - Publications

The following are publications and presentations arising partly or wholly from the work outlined in this thesis;

Ahmad-Annuar A, Shah P, Hafezparast M, Hummerich H, Witherden AS, Morrison KE, Shaw PJ, Kirby J, Warner TT, Crosby A, Proukakis C, Wilkinson P, Orrell RW, Bradley L, Martin JE, Fisher EM. No association with common Caucasian genotypes in exons 8, 13 and 14 of the human cytoplasmic dynein heavy chain gene (DNCHC1) and familial motor neuron disorders. *Amyotroph Lateral Scler Other Motor Neuron Disord.* 2003;4:150-7

Wilkinson PA, Crosby AH, Turner C, Bradley LJ, Ginsberg L, Wood NW, Schapira AHV, Warner TT. A clinical, genetic and biochemical study of SPG7 mutations in hereditary spastic paraplegia. *Brain.* 2004;127:973-80.

Bradley MD, Bradley LJ, deBelleRoche J, Orrell RW. The inheritance of familial ALS. *Neurology.* 2005;64(9):1628-31.

Bradley LJ, Taanman J-W, Muddle J, Orrell RW. Investigation of intercellular differences of mitochondrial protein expression in ALS. *Amyotroph Lateral Scler Other Motor Neuron Disord.* 2003:4(suppl 1):87-88. (Poster presented at the International Symposium on ALS/MND, Milan) 2003.

Bradley LJ, Taanman J-W, Muddle J, Orrell RW. Mitochondrial abnormalities in the remote tissues of patients with ALS. Abstract presented at European Symposium on ALS, Nice 2004.

Bradley LJ, Taanman J-W, Muddle J, Orrell RW. Conflicting evidence for mitochondrial dysfunction in patients with ALS. *Amyotroph Lateral Scler Other Motor Neuron Disord.*

2004:5(suppl 2):70. (Poster presented at the International Symposium on ALS/MND, Philadelphia) 2004.